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THE CHEMISTRY OF CAPREOMYCIN

A THESIS

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by

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GLOSSARY OF ABBREVIATIONS

Ala	Alanine
Capr	Capreomycidine
Dapa	α,β-Diaminopropionic Acid
DNP	2,4-Dinitrophenyl
β-Lys	β-Lysine
Nmr	Nuclear Magnetic Resonance
Ser	Serine
STP	Standard Temperature and Pressure
TLC	Thin Layer Chromatography
Uv	Ultraviolet

SUMMARY

Capreomycin is an antibiotic used in the treatment of tuberculosis cases that do not respond to other treatment. Capreomycin sulfate is a white powder, insoluble in most organic solvents, and soluble in water. In water it has λ mas 266 mμ in the ultraviolet spectrum. The antibiotic is a mixture of four peptides called capreomycins IA, IB, IIA, and IIB.

It has been reported that the major component of this mixture, capreomycin IB, releases alanine, β-lysine, diaminopropionic acid, capreomycidine, and ammonia in the ratio 1:1:2:1:1. Previous research concerning the sequence of the amino acids in the capreomycin IB peptide led to the partial structures I and II.

I II

Since capreomycin IB contains two moles of diaminopropionic acid and the dipeptide diaminopropionyl-capreomycidine, it was not possible to determine if this dipeptide resulted from a partial structure such as I or a partial structure such as II.

An investigation on the nature of the chromophore in capreomycin led to the proposal that a dehydroalanine derivative containing a vinylurea unit was responsible for the ultraviolet absorption. Viomycin, a related antibiotic, appears to have the same chromophore as capreomycin. In this laboratory, a sample of viomycin, which does not release urea

on hydrolysis, but which still has the same ultraviolet characteristics as viomycin containing urea, has been obtained. It appears doubtful, therefore, that urea is involved in the chromophores of viomycin and capreomycin.

Further research on the nature of the chromophore and on the sequence of the amino acids in capreomycin was required for structure elucidation.

The first objective of this research was to separate the mixture of capreomycins. It was, therefore, necessary to develop a qualitative method of analysis to distinguish between the various components. Thin layer chromatography on silica gel plates was found to be useful for this purpose.

In an effort to separate the capreomycin mixture, gel chromatography using several types of gels was used, but no separation could be obtained. A combination of gel and ion exchange chromatography using DEAE-Sephadex yielded material which appeared to be pure according to qualitative TLC analysis. Amino acid analysis of this material indicated that it was still a mixture of capreomycins IA and IB. It was, however, possible from this analysis to conclude that capreomycin IB contained alanine, β-lysine, diaminopropionic acid, capreomycidine, and ammonia in the ratio 1:2:3:1:2.

End-group analyses on capreomycin revealed that β-lysine was the N-terminal amino acid in the peptide. The C-terminal amino acid did not correspond to any of the amino acids known to occur in capreomycin. It was not possible to identify this compound. pKa values obtained for capreomycin IB indicated that the α-amino group of a diaminopropionic

acid unit might be free also. It was, however, not possible to confirm this suspicion by experiment.

Acid hydrolysis of capreomycin for a short time released urea, but none of the amino acids from capreomycin, leading to the formation of desureacapreomycin. End-group analyses on desureacapreomycin showed that no C-terminal amino acid was created by the release of urea, and that urea was therefore not present as an ureide in capreomycin.

Hydrolysis of capreomycin in 6 N hydrochloric acid at room temperature over a period of several weeks led to the formation of a complex mixture of peptides. During this reaction β-lysine was the only amino acid released. Attempts to separate the mixture of peptides by means of electrophoresis and ion exchange chromatography were not successful.

Hydrolysis of capreomycin in 1 N sodium hydroxide also led to the formation of a mixture of peptides. Urea was released during this reaction and the ultraviolet chromophore of the molecule disappeared. Since it was convenient to remove barium ions by precipitation as barium carbonate, capreomycin was hydrolyzed with a barium hydroxide solution. After hydrolysis, barium ions were removed as barium carbonate, and the hydrolysate was treated with copper(II) hydroxide. The copper complexes of the peptides formed in this way were subjected to gel chromatography but no pure peptides could be isolated.

In another attempt to obtain peptides, capreomycin was hydrolyzed in concentrated hydrochloric acid. The resulting mixture of peptides was converted into their DNP-ester derivatives, and they were separated by a combination of column chromatography and thin layer chromatography.

The purified compounds obtained in this way were subjected to mass spectrometry. Using this method, it was possible to identify the peptides alanyl-β-lysine, diaminopropionyl-alanine, and β-lysyl-diaminopropionic acid.

In an effort to distinguish between peptides originating from the N-terminal β-lysine unit and those from the non-terminal β-lysine unit, DNP-capreomycin was partially hydrolyzed by acid. The hydrolysate was treated with ethanol/hydrogen chloride and was then acetylated. Chromatography over silicic acid was used to separate the mixture obtained. The first yellow band eluted was subjected to mass spectrometry. Using this method it was possible to identify the compound diacetyldiaminopropionylalanyl-(ε-DNP)-β-lysine ethyl ester. This revealed that the ε-amino group of the β-lysine unit that was not N-terminal was also free in capreomycin.

Hydrogenation of capreomycin consumed four equivalents of hydrogen. During this reaction urea was released and the ultraviolet chromophore disappeared. Hydrolysis of hydrogenated capreomycin released two equivalents of alanine, whereas capreomycin itself upon acid hydrolysis yielded only one equivalent of alanine. This indicates the presence of a dehydroalanine derivative or a unit convertible to alanine by hydrogenolysis in capreomycin. End-group analyses on hydrogenated capreomycin showed that this alanine derivative was neither N-terminal nor C-terminal.

The information gained through this research still does not show the complete amino acid sequence of capreomycin. The chromophore is also unaccounted for.

It was thought that an ideal way to obtain the complete structure of capreomycin would be through an X-ray crystallographic analysis. In an attempt to obtain crystals of heavy atom derivative of capreomycin for this purpose, a copper complex of capreomycin was prepared and purified by means of gel chromatography. It was, however, not possible to crystallize this compound.

CHAPTER I

INTRODUCTION

Isolation and Biological Activity

Capreomycin, a peptidic antibiotic, was isolated from the fermentation broth of *Streptomyces capreolus* and was first described in 1960 (1). The antibiotic was found to be active against many gram positive and gram negative bacteria and also against mycobacteria (2,3,4). It also showed activity in the treatment of leprosy in mice (5) and was found to be active against several *Nocardia* species isolated from human illnesses (6).

In tests against *Mycobacterium tuberculosis*, capreomycin did not show a definite pattern of cross-resistance with streptomycin, kanamycin, cycloserine, isoniazid, *p*-aminosalicylic acid, or ethionamide (3,7). Strains of *M tuberculosis* that were resistant to capreomycin were also resistant to viomycin; however, strains resistant to viomycin were not consistently cross-resistant to capreomycin (7).

Later work revealed that capreomycin was actually a mixture of four different peptides which were called capreomycins IA, IB, IIA, and IIB (8). *In vitro* results showed that the capreomycin IA and IB were approximately 2.5 times more active against *Klebsiella pneumonia* than were capreomycins IIA and IIB. Capreomycins IA and IB were approximately seven times more active against *Mycobacterium butyricum* than the capreomycin II's.

Clinical studies with capreomycin revealed that mice and dogs are more susceptible to its toxic effects than cats. Renal injury resulted from treatment with capreomycin in those animals susceptible to the drug. Prolonged treatment also led to auditory injury in dogs (9). In preliminary studies with humans, capreomycin was used with sodium p-aminosalicylate against pulmonary TB. Toxicity was infrequent, but when observed was chiefly loss of hearing (10).

In spite of its toxic reactions, capreomycin still finds clinical application in cases where strains of the tuberculosis micro-organism has become resistant to streptomycin.

Properties of the Intact Molecules of the Capreomycins

Capreomycin* is a white solid, soluble in water and relatively insoluble in most organic solvents (11). Some of the physical properties which have been reported for the various members of the capreomycin group of antibiotics and for the mixture are summarized in Table 1.

An exact molecular weight and a molecular formula is not known for any of the capreomycins. The reason for this is that it is extremely difficult to obtain these compounds pure enough for reliable analyses.

Capreomycin gives positive ninhydrin, Folin Ciocalteu, and Lowry tests and negative anthrone, Bial, Tauber, Ehrlich indole, and hydroxamic acid tests (12).

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*In this document the word "capreomycin" will refer to the mixture of capreomycins IA, IB, IIA, and IIB.

Table 1. Physical Properties of the Capreomycin Group of Antibiotics

Compound	pKa values (water)	Apparent Mol.weight	U.V. max(mμ)	ε	$E^{1\%}_{1cm}$	[α]	IR max cm^{-1}	Nmr τ	Ref.
Capreomycin IA (sulfate)	3.2, 6.3, 8.2, 10	1115	266 (water)	26,650	-	-	3280, 2941, 2850, 1671, 1520, 1502, 1463, 1377, 1228, 1100	-	8,13
Capreomycin IB (sulfate)	3.5, 6.3, 8.2, 10	1005	266 (water)	24,050	-	-	"	-	8
Capreomycin IB (crystalline free base)	4.2, 6.7, 8.7, 10.6	698	268 (acid)	-	363	-20.56°		-	13
"			287 (base)	-	239			-	13
								1.9 (D_2O)	15
Capreomycin IIA (sulfate)	1.9, 6.3, 8.1, 11	770	266 (water	26,400	-	+13.2°	"	-	8
Capreomycin IIB (sulfate)	1.9, 6.3, 8.1, 11	790	266 (water)	26,950	-	-26.6°	"	-	8

Total Degradation of Capreomycin

Capreomycin released the amino acids serine, alanine, β-lysine (β, ε-diaminocaproic acid), capreomycidine (α[2 imino-hexahydro-4-pyrimidyl]glycine), diaminopropionic acid, and also ammonia upon acid hydrolysis.* It was, therefore, concluded that the capreomycins are peptide antibiotics (8). The amino acid content of the capreomycins was determined quantitatively and the ratio of the amino acids were calculated on the assumption that each peptide contained a single capreomycidine unit (8). The results are given in Table 2. This table also contains the amounts of ammonia released from each of the capreomycins through acid hydrolysis (13).

Capreomycidine is an amino acid unique to the capreomycins. On the basis of its physical and spectral properties capreomycidine has been shown to be α-(2-iminohexahydro-4-pyrimidyl) glycine (14). This amino acid could also be considered to be a cyclic arginine where cyclization took place between the β-carbon and the terminal amine of the quanidino moiety of arginine.

A compound containing a similar cyclic arginine moiety is viomycidine, an amino acid obtained from acid hydrolyses of viomycin. Bycroft and coworkers suggested that viomycidine is formed from a hydroxy-capreomycidine unit in the viomycin molecule since hydrogenated viomycin on acid hydrolysis gave rise to capreomycidine instead of viomycidine (15). Since the absolute stereochemistry of viomycidine is well established (16,17), this relationship between viomycidine and capreomycidine also

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*An unidentified ninhydrin positive compound was also noted (8).

revealed the absolute stereochemistry of capreomycidine.

The gross structure of capreomycidine has been confirmed by synthesis (18).

Table 2. Amino Acid Composition of the Capreomycins

	Ser	Ala	Dapa	β-Lys	Capr	Ammonia
Capreomycin IA	1.0	0.1	1.7	1.2	1.0	1.0
Capreomycin IB	0.1	0.7	1.8	1.1	1.0	1.0
Capreomycin IIA	1.0	0.06	2.3	0.05	1.0	1.0
Capreomycin IIB	0.25	1.1	2.3	0.05	1.0	1.0

Other natural products which contain cyclic arginine moieties are stendomycidine (19), an amino acid found in stendomycin (20), and tetrodotoxin (21).

Partial Degradation of Capreomycin

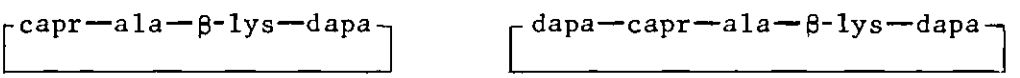
Herr and Redstone reported that the commonly occurring proteolytic enzymes did not degrade capreomycin IB (8). Mild acid hydrolysis, on the other hand, released one equivalent of urea and it was found that the production of urea was kinetically related to the disappearance of the uv chromophore (15).

Treatment of capreomycin with acid or base led to the destruction of the uv chromophore. Destruction of the chromophore led to a parallel decrease in the *in vitro* microbiological activity of the antibiotic (8).

A partial acid hydrolysis of capreomycin IB with concentrated hydrochloric acid at 70° for 65 hr yielded a mixture of peptides (8).

These peptides were purified by means of electrophoresis. The major components obtained were further identified by quantitative and qualitative amino acid analysis as well as by end-group analysis of each fragment. The peptides alanyl-β-lysine, β-lysyl-diaminopropionic acid, diaminopropionyl-capreomycidine, and capreomycidyl-alanine were identified in this way. The existence of the dipeptide unit diaminopropionyl-capreomycidine in capreomycine was confirmed by Bycroft and coworkers (15).

Since capreomycin IB contains two moles of diaminopropionic acid and the dipeptide diaminopropionyl-capreomycidine, it was not possible to determine if this dipeptide resulted from a partial structure such as I or a partial structure such as II.



I II

Studies on the Nature of the Chromophore in Capreomycin

It has been found that in water and acidic solutions all of the capreomycins absorb uv light at λ max 266 mμ (Table 1). The position and intensity of this absorption seems to be dependent on the pH of the solution since capreomycin IB shows a λ max at 287 mμ ($E^{1\%}_{1cm}$ 239) in a basic aqueous solution (13). A λ max at 290 mμ (ε 15,000) has also been mentioned for capreomycin IB in a basic solution (22).

The antibiotic viomycin resembles the capreomycins in many aspects. For instance, viomycin has a λ max at 268.5 mμ (ε 23,000) which shifts to 282.5 mμ (ε 14,500) in 0.1 N aqueous sodium hydroxide

(23). Studies of the variation of the position of absorption with pH showed two isosbestic points at 235 mμ and 281 mμ. Analysis of these data indicated that one dissociating group, pKa 12.4, was involved in the chromophore of viomycin (24). Viomycin, like capreomycin, releases one equivalent of urea when treated with dilute hydrochloric acid. The rate at which urea was released from viomycin was also found to be equivalent to the rate at which the chromophore disappeared (25).

From these data it can be seen that there exists a close relationship between the chromophore of the capreomycins and that of viomycin. Therefore it seems appropriate also to review the work done on the chromophore of viomycin.

Bycroft et al, reported (25) that hydrogenation of viomycin led to the formation of urea and a perhydroviomycin derivative. The hydrogenated material did not show an absorption in the uv region of the spectrum and it did not possess a low field proton (τ 1.9) in the nmr like viomycin. Complete acid hydrolysis of hydrogenated viomycin released all of the amino acids* found in viomycin plus one equivalent of alanine, an amino acid not originally present in viomycin. Oxidation of viomycin with potassium permanganate destroyed the chromophore and afforded urea and oxalic acid (25).

On the basis of these findings Bycroft and coworkers proposed structure III for the chromophore in viomycin (25). These researchers also stated that similar conclusions had been reached on the nature of the chromophore in capreomycin.

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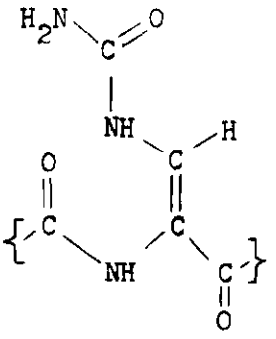
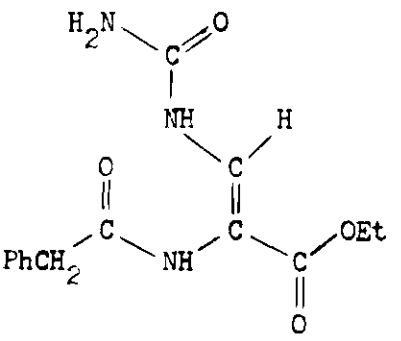
*The fact that capreomycidine instead of viomycidine is obtained from hydrogenated viomycin has been mentioned elsewhere.



III



IV

Recently a model compound (structure IV) containing this proposed chromophoric unit was synthesized (22). The spectral properties of this compound was found to be similar to those of viomycin and capreomycin. Also, the pKa value of 12.6 was in close agreement with the pKa's of the two antibiotics.

Hydrogenation of compound IV followed by acid hydrolysis afforded alanine, while oxidation with potassium permanganate led to the formation of formyl urea, as was observed with viomycin.

In conflict with the proposal that structure III represents the chromophore is the report (26) that a sample of viomycin has been obtained which does not release urea upon acid hydrolysis. It was also recently found that hydrogenation of this material does not release urea (27).

This recently produced viomycin, however, still has the same biological activity as viomycin that contains urea. It also has the same uv absorption in acidic and basic solutions and has a low field proton (τ 1.9) in the nmr equivalent to urea-containing viomycin.

In the light of these findings it is doubtful that structure III represents the chromophore of viomycin. Since the chromophore in

capreomycin appears to be very similar to the chromophore in viomycin, there are also some doubt that structure III represents the correct chromophore of capreomycin.

This research was aimed at obtaining more detailed information about the sequence of amino acids in capreomycin. It was planned to obtain this knowledge about the amino acid sequence through an investigation of the peptides formed during partial hydrolyses of capreomycin and through end-group analyses of capreomycin. It was also the aim of this investigation to learn something about the chromophore in capreomycin.

CHAPTER II

EXPERIMENTAL

Apparatus and Techniques

Gel Chromatography

The gels Sephadex G-10, G-15, and G-25 (Pharmacia Fine Chemicals, Inc.) were used for the separation of the peptides, copper complexes, and hydrolysis products of capreomycin. These gels were equilibrated with solvent and used as prescribed by the manufacturer (28). In all cases the gels were packed as a slurry into a column and allowed to settle under the influence of gravity.

Ion Exchange Resins

Ion exchange resins were used for the conversion of peptide salts into their free base analogues, exchange of the anions in peptide salts, and for the separation of amino acids and peptides.

Resins used and treated as described previously (26) were Amberlite IR-400 (Rohm and Haas Co.), a strong basic anion exchange resin, Dowex 50X8 (Dow Chemical Co.), a strong cation exchange resin, and CM-Sephadex-C-25 (Pharmacia Fine Chemicals, Inc.), a weakly acidic cation exchange polydextran material.

The weakly basic anion exchange polydextran DEAE-Sephadex-A-25 was also used. This material was supplied by Pharmacia Fine Chemicals, Inc. in the chloride form. The chloride could be displaced by other anions in a way described by the manufacturers (29).

Chelex 100 (Bio Rad Laboratories) is a resin capable of complexing with heavy metal cations. It was used in the cupric ion complexed form in an attempt to separate the copper complexes of capreomycin from uncomplexed capreomycin. The sodium form of this resin was treated with an excess of a concentrated cupric chloride solution until no more cupric ions were taken up by the resin. It was then washed thoroughly with distilled water until the wash water did not give a precipitate when treated with a silver nitrate solution.

Thin Layer and Paper Chromatography

Paper chromatography was used as described previously (30). Thin layer chromatographic analyses of the peptides and amino acids obtained from capreomycin were done on plates of silica gel HF_{254} (Brinkman Instruments Inc.). The plates were prepared by spreading a 0.25 mm layer of a slurry of silica gel in water on glass plates by means of the spreader developed by Stahl (31). The slurry was made by thoroughly stirring 30 g of silica gel into 90 ml water until no lumps or bubbles remained. As recommended by M. Brenner *et al* (32) the plates were left to dry in the open air. After the samples were introduced onto the plates activation was done for five minutes at 130^{o}. The plates were then allowed to cool in the vapors of the solvent in the development chamber for at least 20 minutes before development.

Solvents that were frequently used for development of the plates were abbreviated as follows: BAAAW, *n*-butyl alcohol: acetone: acetic acid: 5% aqueous ammonia: water:: 9:3:2:2:4; MAWD, methanol: acetic acid: water: dimethylsulfoxide:: 8:3:15:10; CMA, lower phase of chloroform: methanol: 17% aqueous ammonia:: 2:2:1.

Visualization of spots on the chromatograms were effected by viewing the fluorescent silica gel HF_{254} plates in a dark room under short wavelength ultraviolet light. Ninhydrin, Weber, and p-dimethylaminobenzaldehyde spray reagents were also frequently used. There spray reagents were prepared and applied as previously described (30,33).

Electrophoresis

A Beckman Model R electrophoresis apparatus, Beckman no. 320046 and no. 319328 paper strips as well as cellulose acetate strips (Millipore Corp.) were used. Buffer systems that were found useful were a 1.5% sodium tetraborate solution adjusted to pH 9.1 and a 0.005 M ammonium formate buffer of pH 9.4.

All electrophoretic analyses were done at a constant voltage. The voltage, distance of migration from origin, direction of migration, buffer used and the time of the run were recorded for each analysis.

Detection of the bands on the strips were done in the same way as in thin layer and paper chromatography.

Instruments

Ultraviolet spectra were obtained using a Cary Model 14 recording spectrophotometer. A Varian A-60D 60 megacycle n.m.r. instrument was used with TSS as an internal standard to obtain n.m.r. spectra.

Mass spectra were recorded with the help of a Varian M66 Mass Spectrometer. The automatic fraction collector used with column chromatography was a GM Instrument Company, Inc. Model VE-2002-B-24. A Rinco rotary evaporator was used to remove solvents from solutions under reduced pressure.

Optical rotations were taken with a Bellingham and Stanley Model

397619 polarimeter. Measurements of pH were done with a Leeds and Northrup no. 7401 pH meter.

Attempted Purification of Capreomycin

Crude capreomycin (sample no. 566-532AD-201)* was obtained from Eli Lilly and Co. This material was subjected to the following quantitative and qualitative chromatographic analyses in an effort to purify and characterize the compounds in the mixture.

Thin Layer Chromatography

Two microliters of a 0.2% (w/v) aqueous solution of crude capreomycin were applied to a TLC plate and developed with BAAAW. After spraying with ninhydrin, four components with R_F values 0.03, 0.13, 0.2, and 0.25 became visible. The spot at R_F 0.2 was the largest and could obviously be attributed to the major component.

When similar analyses were carried out, it was found that the R_F values of the different components varied somewhat depending on several parameters. The separation between spots, however, was almost always satisfactory.

Electrophoresis

One cell of the electrophoresis apparatus was filled with eight paper strips (Beckman no. 319328). An aqueous solution containing 15 mg of the capreomycin mixture was applied as a uniform band in the middle of each paper. A pH 9.1 sodium tetraborate buffer was used for the analyses which were done at a constant voltage of 175 volts for 4 hr.

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*Two samples of capreomycin (566-532AD-201 and 566-532AD-200) were obtained from Eli Lilly and Co. Unless specified otherwise, the sample 566-532AD-201 was used.

The papers were then dried and sprayed with a ninhydrin solution. Four bands that migrated 5 cm, 8 cm, 9.25 cm, and 10 cm towards the negatively charged electrode were observed. The band at 5 cm seemed to be the major component.

When samples of pure capreomycin IA, IB, and IIA were compared with the crude capreomycin on similar electrophoretic analyses, it was found that all migrated comparable distances from the origin. It was also observed that if the amount of crude capreomycin on the paper strips was increased, the four bands tended to overlap. For these reasons, it appeared that TLC provided a better qualitative analyses of the components in capreomycin.

Gel Chromatography of Capreomycin on Sephadex G-10

Dry Sephadex G-10 was equilibrated with water at room temperature for 24 hr. The suspension that resulted was poured into a column, 210 cm x 3 cm, and allowed to settle. The Sephadex filled the column to a height of 193 cm.

A 3.8 g sample of crude capreomycin sulfate was dissolved in 5 ml of water and introduced onto the column. The material was washed on the column with an additional 5 ml of water. Further development was done with water at a rate of 2 ml/min, and 10 ml fractions were collected.

Acetone was added to each fraction; a white precipitate indicative of the presence of capreomycin appeared in fractions 25-46. These fractions were concentrated under reduced pressure on a rotary evaporator and analyzed by TLC using the BAAAW solvent system and ninhydrin as a spray reagent.

A compound which showed a spot at R_F 0.0 occurred in fractions

25-40. A compound (R_F 0.06) was found in fractions 25-29, and in fractions 34-40 a compound with a R_F value of 0.15 was present. The major component had a R_F of 0.08 and was located in all fractions between 25 and 46.

These TLC data showed that considerable overlap of the various components occurred and that no satisfactory separation took place.

When fractions 25-46 were combined and evaporated to dryness 3.6 g of material could be recovered.

Chromatography of Capreomycin on Sephadex G-15

Sephadex G-15 was equilibrated with an excess of water for three hours at room temperature, then packed as a slurry in a glass column and allowed to settle under the influence of gravity. A column 94 cm x 1.5 cm of Sephadex was obtained in this way.

Three hundred milligrams of capreomycin sulfate was dissolved in 2 ml of water and washed onto the column with additional small portions of water. Further elution was done also with water at a flow rate of about 1 ml/min. By means of a fraction collector 5 ml fractions were collected.

Capreomycin was detected by spotting a small amount of each fraction on a silica gel HF_{254} plate. When the plate was viewed in ultraviolet light those fractions that contained capreomycin appeared as dark spots against a fluorescent background. In this manner, it was found that capreomycin occurred in fractions 20-30. A TLC analyses with BAAAW showed that two components, one at R_F 0.11 and one at R_F 0.18 were present in fractions 20-30. Although no clear separation was observed, it was noted that fractions 20-24 contained relatively more of the low

R_F component while it appeared that the high R_F compound was concentrated in fractions 25-30.

On the basis of these results, further attempts to purify capreomycin by means of gel chromatography was abandoned.

Ion Exchange Chromatography of Capreomycin on DEAE-Sephadex A-25

The chloride form of DEAE-Sephadex A-25 was converted into its acetate form by repeatedly washing the anion exchanger with a concentrated solution of sodium acetate. A solution consisting out of two volumes methanol, one volume acetone, and one volume water (2:1:1) was made 0.2 M in pyridinium acetate and was used for further extensive washing of the ion exchanger. Finally the Sephadex was suspended in this solvent and was poured into a column 100 cm x 3 cm. After it was allowed to settle the solid filled the column to a height of 68 cm.

Four grams of crude capreomycin sulfate was dissolved in 3 ml of water and carefully poured onto the column. This solution was drained into the column and then further eluted first with 10 ml of water and then with 10 ml of a 1:1 mixture of water and the pyridinium acetate 2:1:1 solvent. The 2:1:1 solution was subsequently used for further development of the column.

After 200 ml of the eluate was collected, the composition of the solvent was changed to a mixture of 3:2:3 (by volume) of methanol, acetone, and water which contained 0.4 M pyridinium acetate.

The flow rate through the column was maintained at 8 ml/3 min and 8 ml fractions were collected. Acetone was added to the fractions and a white precipitate appeared in fractions 32-68. Each one of these fractions was concentrated under reduced pressure on a rotary evaporator

and then subjected to TLC analyses using the BAAAW solvent system.

Fractions 35-56 contained only the major component of the original mixture which appeared as a ninhydrin positive spot at R_F 0.11; consequently these fractions were combined and evaporated to dryness to obtain 1.44 g of purified material.

Gel Filtration of Purified Capreomycin

It was found that the material obtained from ion exchange chromatography on DEAE-Sephadex still contained pyridinium acetate and needed further purification. Therefore, a sample of 0.37 g of this material was dissolved in a minimum of water and eluted through a Sephadex G-10 column 44 cm x 1 cm, which had been equilibrated with water. Elution was done at a flow rate of 1.8 ml/5 min and 1.8 ml fractions were collected.

Through precipitation with acetone, it was observed that capreomycin occurred in fractions 8-13. Each of these fractions were analyzed by TLC using BAAAW as the solvent for the development of the plates. Only the leading and trailing fractions (8 and 13) were found to contain impurities. Fractions 8 and 13 were consequently discarded, the rest of the acetone insoluble fractions were combined and evaporated to dryness. The amount of material regained was 0.115 g (32%).

Amino Acid Analysis

A sample of capreomycin purified, as described above, was hydrolyzed in a sealed tube at 105° for 24 hours and then analyzed on a Moore-Stein amino acid analyzer.* The results indicated that the peptide

*This analysis was kindly performed by Eli Lilly & Co.

contained alanine, diaminopropionic acid, β-lysine, capreomycidine, and ammonia in the ratio 1:3:2:1:2.

Attempted Crystallization of Capreomycin

The attempt to crystallize capreomycin was based on the method of G. Wild from Eli Lilly and Co. (34).

Amberlite IR-400 was converted into its hydroxide form and packed to form a column 12 cm x 1 cm. A 50 mg sample of purified* capreomycin was dissolved and eluted through this column with water. An aliquot of the eluate was tested with barium chloride and found to be free of sulfate. The eluate was subsequently evaporated to dryness under reduced pressure at 55^{o} to give 34 mg of glassy material. This material was dissolved in 1.2 ml of water, and methanol was added until the solution became cloudy. Enough water to clear up the cloudiness was added, and this was followed by addition of 1-propanol until a milkiness just appeared. After leaving the solution at room temperature overnight, no crystallization could be observed. When the solution was placed in a refrigerator, a sirupy white precipitate quickly formed.

Capreomycin was then converted into its stoichiometric sulfate by concentrating the solution of free base almost to dryness and then eluting it through a IR-45($SO_4^{=}$) column. All attempts to crystallize the sulfate salt failed.

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*This material was the same as the sample used for the amino acid analyses.

N-Terminal Amino Acid Analysis of Capreomycin

Preparation of the DNP-Derivative of Purified Capreomycin

A 50 mg sample of capreomycin sulfate, obtained from the DEAE-Sephadex purification procedure, was dissolved in 5 ml water. The pH of this solution was adjusted to 10 by means of a dilute potassium hydroxide solution. The solution was then stirred vigorously by means of a magnetic stirrer while 0.5 ml of 2,4-dinitrofluorobenzene was added dropwise over a period of 1.5 hours. Using pH paper, the pH of the reaction mixture was checked every 30 minutes and maintained at 10.

After three hours, the solution was acidified with hydrochloric acid, 5 ml of ethanol was added, and the precipitate that formed was filtered. The precipitate was washed once with 3 ml of water and then twice with 5 ml portions of ethanol. When dried, 53 mg of a yellow powder remained. This material could not be crystallized.

Preparation of the DNP-Derivatives of Crude Capreomycin

In a larger scale preparation 9.5 g of capreomycin (ca. 0.01 mole)*, 5.04 g of sodium bicarbonate (0.06 mole), and 4.65 g of 2,4-dinitrofluorobenzene was weighed into an Erlenmeyer flask. The mixture was suspended in 35 ml of water and stirred overnight using a magnetic stirrer.

After adding 3 ml of concentrated hydrochloric acid, the mixture was then filtered and the precipitate washed two times with 10 ml portions of water followed by one washing with acetone. It was then dried in the atmosphere to yield 8.31 g of a powdery yellow substance which

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*The molecular weight of capreomycin was taken as 950.

again could not be crystallized.

Thin Layer Chromatography of the DNP-Derivatives of Capreomycin

The DNP-derivatives obtained from crude and purified capreomycin were compared by TLC using BAAAW on silica gel HF_{254} plates. Two yellow spots, one at R_F 0.5 and one at R_F 0.79 were observed for the crude material. The spot at R_F 0.5 seemed to represent a small quantity of a minor component of the DNP-capreomycin mixture. A single yellow spot at R_F 0.79 was observed for the pure material.

Preparation of DNP-Derivatives of the Amino Acids found in Capreomycin

DNP-L-serine, bis-DNP-β-lysine, and bis-DNP-diaminopropionic acid were prepared by Kellogg (30) according to the method of Rao and Sober (35). These samples were available for comparison studies.

DNP-Alanine. Two hundred milligrams of the hydrochloric acid salt of alanyl ethyl ester was dissolved in 10 ml of water and treated with an excess of sodium bicarbonate. The solution was stirred using a magnetic stirrer, and 0.2 ml of 2,4-dinitrofluorobenzene was added over a period of 15 min. After 1 hr, the reaction mixture was extracted with 20 ml of ethyl acetate, and the extract was washed first with a 1 N potassium hydroxide solution and then with dilute hydrochloric acid. It was then washed with a dilute aqueous sodium bicarbonate solution and was dried over calcium chloride. The ethyl acetate was removed using a rotary evaporator, the residue was dissolved in chloroform, and an excess of cyclohexane was added. The mixture was left overnight in a refrigerator. The yellow needles which crystallized had a mp 103° (corr). The yield was 256 mg (69%).

A small portion of the DNP-alanyl-ethyl ester was hydrolyzed with

6 N hydrochloric acid on a steambath for 1 hr. The hydrochloric acid was removed by evaporation under reduced pressure, and the residue was dissolved in 10 ml of a dilute sodium hydroxide solution. This solution was extracted with ethyl acetate, and the organic phase was discarded. The aqueous solution was thereupon acidified and once more extracted with ethyl acetate. After the extract had been dried over calcium chloride, it was evaporated to dryness.

DNP-alanine was obtained as a yellow powder which showed one spot on TLC. Table 3 gives RF values and the solvent systems used.

α-Mono DNP-Capreomycidine. A mixture of 50 mg of the hydrochloric acid salt of capreomycidine, 0.2 ml of 2,4-dinitrofluorobenzene, and an excess of sodium bicarbonate was suspended in 50 ml of water and stirred vigorously for 1 hr. A yellow precipitate formed that was filtered and dissolved in a 1:1 mixture of 6 N hydrochloric acid and methanol. The solution was then evaporated to dryness and was dissolved in methanol. When left overnight in a refrigerator, the methanolic solution yielded a small amount of white crystals. These were filtered, and the filtrate was evaporated to yield 35 mg of a yellow powder. Using TLC, this material was shown to contain several components. Table 3 contains the R_F values and the solvent systems used.

α-Mono-DNP-Diaminopropionic Acid. A copper complex of diaminopropionic acid was prepared as follows: 500 mg of the hydrochloric acid salt of diaminopropionic acid was dissolved in 40 ml of water and stirred with 1 g of freshly precipitated copper (II) hydroxide for 6 hr after which the solution was filtered. With the help of a rotary evaporator, the water was removed from the filtrate under reduced pressure to yield

Table 3. TLC Comparison of DNP-Derivatives Obtained from 12N Hydrochloric Acid Hydrolysis of DNP-Capreomycin with DNP-Amino Acid Standards

Sample	Solvent System*	R_F Values
Precipitate formed during 12N hydrochloric acid treatment of DNP-capreomycin	a	0.87
	b	0.92
	c	0.57
Bis-DNP-β-lysine	a	0.87
	b	0.93
	c	0.57
Ethyl acetate extract from the 12N hydrochloric acid hydrolysis of DNP-capreomycin	a	0.87
	b	0.92
	c	0.57
Water soluble DNP-component from acid hydrolysis of DNP-capreomycin	a	0.21
	b	0.03
	d	0.63
Bis-DNP-diaminopropionic acid	a	0.83
	b	0.07
	c	0.53
α-Mono-DNP-diaminopropionic acid	a	0.92
	b	0.54
	c	0.51
β-Mono-DNP-diaminopropionic acid	a	0.73
	b	0.01
	c	0.37
	d	0.66,0.69,0.80
DNP-alanine	a	0.92
	c	0.54
	d	0.81
DNP-serine	a	0.92
	b	0.09
	d	0.75
DNP-capreomycidine	a	0.39,0.48
	b	0.0
	c	0.34,0.39,0.42,0.44
	d	0.61

Table 3 continued

2,4-Dinitrophenol	a	0.94
	b	0.63
2,4-Dinitroaniline	a	0.92
	b	0.96

*(a) Water
(b) Acetone, water, chloroform, p-dioxane, benzene: 3:1:2:2:2.
(c) Acetone, water, chloroform, p-dioxane: 3:1:2:2.
(d) BAAAW.

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350 mg (89%) of a dark blue copper complex.

A 100 mg sample of this copper complex was dissolved in 30 ml of water, and 100 mg sodium bicarbonate and 0.11 ml of 2,4-dinitrofluorobenzene was added and then the solution was stirred for 2 hr. Hydrochloric acid was added until the solution reached pH 2, following which the solution was evaporated to dryness under reduced pressure.

The residue was extracted with ethanol and the material thus obtained was further purified by chromatography on a 32 cm x 1 cm silicic acid column. Ethyl acetate was used to elute the DNP-derivative; the major band was collected, and the solvent was removed to yield 25 mg of a yellow powder. This material showed one spot on TLC. Table 3 gives R_F values and solvents used.

β-Mono-DNP-Diaminopropionic Acid. In a mixture of 5 ml of water and 2 ml of pyridine, a sample of a 0.323 g of the copper complex of diaminopropionic acid (prepared as described above) was suspended and the suspension was stirred using a magnetic stirrer. The solution was thoroughly cooled in an ice bath and 0.35 ml of carbobenzoxychloride was

added over a period of 30 min. The reaction was allowed to proceed for another 15 hr, and the reaction mixture was then evaporated to dryness *in vacuo*. A 95% ethanol solution (15 ml) was then used to wash the residue. After drying the remaining residue *in vacuo*, 310 mg of a white material was obtained. This carbobenzoxy derivative was not purified further but was used directly for the coupling reaction with 2,4-dinitrofluorobenzene.

All 310 mg of the carbobenzoxy derivative was reacted with 0.35 ml of 2,4-dinitrofluorobenzene in 20 ml of water which was adjusted to *p*H 10 with dilute potassium hydroxide. The reaction mixture was stirred for 1 hr and then hydrochloric acid was added until *p*H 2.5 was reached.

Unreacted 2,4-dinitrofluorobenzene as well as 2,4-dinitrophenol were removed from the reaction mixture by repeated washing with diethyl ether; the ether was discarded and the aqueous solution was evaporated to dryness. The product obtained was then dissolved in a mixture of 95% ethanol and glacial acetic acid (9:1 v/v) and was eluted through a silicic acid column with the same solvent mixture. Only the major yellow band was collected. Upon evaporation, a DNP-derivative was obtained that showed only one spot on TLC (Table 3). This DNP-derivative gave a positive ninhydrin reaction and was, therefore, thought to be the β-DNP derivative of diaminopropionic acid.

Complete Acid Hydrolysis of DNP-Capreomycin

A sample of 1 g of the DNP-derivative prepared from crude capreomycin was suspended in 50 ml of concentrated hydrochloric acid in a pressure bottle. The bottle was firmly stoppered and heated on a steam bath for 20 hr. The yellow precipitate which formed during the hydrolysis

was filtered, and the filtrate was extracted three times with a total of 100 ml of ethyl acetate. The combined ethyl acetate extracts were dried over calcium chloride, after which the solvent was removed under reduced pressure. Since the aqueous filtrate retained a yellow color after ethyl acetate extraction, this filtrate was also evaporated to dryness. To characterize the DNP-amino acids in the hydrolysate of DNP-capreomycin, the precipitate, ethyl acetate, and water soluble fractions were compared with known DNP-derivatives by means of TLC. The results of this comparison are given in Table 3.

An analysis of the results contained in Table 3 indicate that the precipitate and the ethyl acetate extract obtained from the DNP-capreomycin hydrolysis were the same as bis-DNP-β-lysine. To confirm this observation, the precipitate was again compared by TLC with bis-DNP-β-lysine using chloroform, ethyl acetate, and methanol each as development solvents. In all three cases the same R_F values were observed for the standard and the precipitate. Subsequently, the precipitate and ethyl acetate extracts were combined to yield 260 mg of the DNP-derivative.

Synethesis of Bis-DNP-β-Lysine Ethyl Ester

The precipitate which formed during the 12 N hydrochloric acid hydrolysis of DNP-capreomycin was dissolved in 75 ml of anhydrous ethanol, and 6 ml of freshly distilled acetyl chloride was added as a source of dry hydrochloric acid (36). After allowing the esterification reaction to proceed at room temperature for two days, the solvent was removed under reduced pressure with a rotary evaporator. The residue was dissolved in ethyl acetate and washed with a 5% sodium bicarbonate

solution. The ethyl acetate solution was then dried over calcium chloride before it was concentrated to about 5 ml.

This concentrated solution was applied to a silicic acid column with the dimensions 62 cm x 1 cm and was eluted with ethyl acetate. Only the major band, which was also the first band to be eluted, was collected and evaporated to dryness. The dried material was dissolved in absolute ethanol and precipitated once with cyclohexane. The precipitate was dried thoroughly in a desiccator under vacuum over sodium hydroxide pellets. A mass spectrum was taken on a small portion of this material.

The results of the mass spectrum are given in Table 4 under compound 1. Only those peaks appearing above m/e 190 are given. Between m/e 190 and m/e 324 a specific amplitude of 5×10^{-12} was used, and the peak at m/e 322 was used as the base peak. Above m/e 324 the specific amplitude was increased by a factor of 10 and the most abundant peak was found at m/e 419. The spectrum was recorded at a temperature of 230°.

Peaks with intensities less than 10% of the intensity of the base peak (or the most abundant peak in the higher mass region m/e 324) were omitted from the table.

Attempted Identification of the Water Soluble DNP-Components obtained from a Complete Acid Hydrolysis of DNP-Capreomycin

The insoluble material from the acid hydrolysate of DNP-capreomycin was removed by filtration. After the filtrate was extracted with ethyl acetate, there remained a yellow color in the aqueous phase. In an effort to identify compounds, the aqueous phase was worked up as follows:

Table 4. Mass Spectra of DNP-Derivatives Isolated from the Acid Hydrolysate of DNP-Capreomycin

Compound	Relative Intensities at m/e =											
	26	27	28	29	30	31	37	38	39	41	42	43
1	-	-	-	-	-	-	-	-	-	-	-	-
2	15	18	17	22	6	-	6	8	20	23	19	100
3	8	16	12	32	-	7	-	-	16	24	-	73
4	-	-	-	-	-	-	-	-	-	-	-	-
	44	45	50	51	52	53	55	57	58	60	61	63
1	-	-	-	-	-	-	-	-	-	-	-	-
2	17	23	10	16	11	7	10	14	27	-	-	7
3	13	25	8	15	13	11	13	20	-	7	7	7
4	-	-	-	-	-	-	-	-	-	-	-	-
	65	67	69	73	77	78	79	81	91	100	101	102
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	6	-	-	13	6	16	-	12	-	-	-
3	8	9	7	6	18	8	19	7	20	-	-	-
4	-	-	-	-	-	-	-	-	-	7	10	8
	103	104	105	106	108	110	111	114	115	116	117	118
1	-	-	-	-	-	-	-	-	-	-	-	-
2	6	6	7	-	-	-	-	-	6	-	-	-
3	7	-	13	-	-	-	-	-	12	-	-	-
4	22	-	18	6	7	19	11	6	-	74	15	12
	119	120	128	129	130	131	133	134	135	142	143	144
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	6	-	-	-
3	6	-	8	7	-	-	6	-	-	-	-	-
4	10	8	7	10	12	6	7	8	-	18	7	8
	145	149	150	152	156	157	158	164	167	174	176	177
1	-	-	-	-	-	-	-	-	-	-	-	-
2	6	7	-	-	-	-	-	-	-	-	-	-
3	11	-	-	-	-	-	-	-	-	-	-	-
4	-	15	17	6	100	22	6	9	8	8	9	6
	179	180	190	191	192	196	197	202	203	204	205	206
1	-	-	27	29	25	88	12	11	15	15	15	22
2	8	-	-	-	-	-	-	-	-	-	52	8
3	-	-	-	-	-	-	-	-	-	-	100	16
4	6	14	23	7	9	47	-	-	-	-	-	6

Table 4 Continued

	208	214	215	216	217	218	220	221	222	232	234	236
1	12	28	13	19	12	-	10	-	41	20	11	40
2	-	-	-	-	-	-	13	-	-	-	-	-
3	-	-	-	-	-	-	27	5	-	-	-	-
4	-	20	9	11	6	-	-	-	-	18	-	20

	242	252	253	259	260	266	278	279	282	288	295	305
1	10	11	-	-	-	26	28	-	25	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	9	-	23	6	4	-	-	25	-	8	9	100

	306	307	309	310	322	323	338	339	340	350	351	361
1	22	-	-	-	100	20	20	22	33	10	14	21
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	> 100	84	30	28	-	80	-	-	-	-	-	-

	362	363	364	365	379	380	381	383	385	389	390	391
1	19	51	25	12	37	17	12	18	10	16	18	10
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-

	401	402	407	419	420	425	435	436	461	462	471	476
1	66	16	12	100	24	21	32	12	46	11	21	23
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-

	489	490
1	12	12
2	-	-
3	-	-
4	-	-

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the water was removed under reduced pressure, and the residue was chromatographed on Whatman no. 17 preparative paper. The solvent system used contained 80 volumes of n-butyl alcohol, 20 volumes of concentrated ammonium hydroxide and five volumes of acetone. After the solvent moved 42 cm down from the point of application, the paper

was removed from the solvent and dried in the air.

Two yellow bands were observed; one had R_F value 0.8 and the other one had R_F value 0.25. The band with R_F 0.25 appeared to be the major component and was eluted from the paper with a dilute hydrochloric acid solution. Using a sintered-glass filter, the eluate was filtered, and the filtrate was evaporated to dryness. The residue obtained in this way was dissolved in dry ethanol which had been saturated with dry hydrogen chloride.

After 24 hr the ethanol/hydrogen chloride mixture was removed under reduced pressure and the residue analyzed on a silica gel thin layer with ethyl acetate. Four different yellow spots which had R_F values of 0.85, 0.64, 0.32, and 0.18 were observed. Subsequently this mixture was subjected to preparative TLC on silica gel with ethyl acetate. Once again, four different bands were observed. The bands which had R_F values of 0.64 and 0.32 appeared to represent the major components of the mixture. These bands were, therefore, scraped from the TLC plates and dried for 4 hr under vacuum in an Abderhalden apparatus at the temperature of boiling pyridine. The samples were not eluted from the silicic acid but were subjected as such for mass spectral analyses.

The mass spectrum obtained for the compound which had a R_F value of 0.64 is given as compound 2 in Table 4. Those peaks which had intensities less than 5% of the intensity of the base peak were omitted from the table. The specific amplitude setting on the mass spectrometer was 5×10^{-12} for this recording and the temperature of the probe was kept at 130°.

The mass spectrum of the compound which had a R_F value of 0.32 is given as compound 3 in Table 4. In this case also only those peaks with relative intensities greater than 5% appears in the table. For this recording, the specific amplitude was 2×10^{-12} and the temperature of the probe was 110°.

In an additional attempt to identify DNP-derivatives in an acid hydrolysate of DNP-capreomycin, 1 g of DNP-capreomycin was hydrolyzed in 50 ml of concentrated hydrochloric acid in a stoppered pressure bottle at 90° for 36 hr. The hydrochloric acid was removed under reduced pressure using a rotary evaporator and the residue was dried in a vacuum desiccator over sodium hydroxide pellets. The dried material was then treated with dry ethyl alcohol which had been saturated with dry hydrogen chloride. After 110 hr the ethanol and hydrogen chloride was evaporated from the mixture using a rotary evaporator. The residue thus obtained was extracted with acetone, and the actone was discarded. The acetone-insoluble residue was suspended in a 5% sodium bicarbonate solution, ethyl acetate was added to this suspension, and the mixture was shaken thoroughly in a separatory funnel. Material which remained insoluble in both phases was removed by filtration, and the ethyl acetate layer was separated from the aqueous phase and dried over calcium chloride.

The material in the ethyl acetate phase was subjected to preparative TLC on silica gel using ethyl acetate as the solvent. A major yellow band which had an R_F value of 0.05 together with several minor components were observed. Using methyl alcohol the major yellow band was extracted, the extract was filtered, and the filtrate was dried <u>in vacuo</u>. Further purification of the residue was achieved by rechromato-

graphing it on preparative silica gel plates with acetone. The major yellow band was extracted from the silica gel with methyl alcohol, the extract was filtered, and the filtrate was evaporated to dryness. A mass spectrum (compound 4, Table 4) was taken on the material thus obtained.

For the recording of this spectrum the specific amplitude on the instrument was set at 2×10^{-12} for the region of the spectrum below m/e 300. The base peak was found at m/e 156.

Above m/e 300 the specific amplitude was increased by a factor of 10. The most intense peak for this region was found at m/e 306; however, this peak went off scale on the recorder and therefore the peak at m/e 305 was used as the most abundant peak.

The whole spectrum was recorded at 110°, only peaks above m/e 100 were given. Peaks with relative intensities less than 5% of the base peak in the mass region below m/e 300 and less than 5% of the most abundant peak above m/e 300 were omitted from the table.

C-Terminal Amino Acid Analysis

Hydrazinolysis of Capreomycin Sulfate

A sample of 100 mg of capreomycin sulfate was sealed with 15 ml of anhydrous hydrazine and 200 mg of hydrazine sulfate in a thick-walled glass tube; the tube was heated at the temperature of boiling acetone for 24 hr. The tube was then cooled, opened, and the contents were dried *in vacuo* in a desiccator over concentrated sulfuric acid. The residue was dissolved in 20 ml of a 5% aqueous sodium bicarbonate solution and the solution was treated with 1 ml of 2,4-dinitrofluorobenzene for 3 hr.

After acidification, the reaction mixture was extracted with ethyl acetate, and the ethyl acetate extract was in turn extracted with a 4% aqueous sodium bicarbonate solution. The material remaining in the ethyl acetate solution was saved and used in the next procedure. The sodium bicarbonate solution was acidified and then extracted with ethyl acetate. Anhydrous sodium sulfate was used to dry this ethyl acetate extract. The solution was then filtered, and the solvent was removed from the filtrate by means of a rotary evaporator.

Using sublimation, most of the 2,4-dinitrophenol present in the residue was removed, and the remaining material was then subjected to a TLC analysis on silica gel. It was found that this material did not correspond to bis-DNP-β-lysine, bis-DNP-diaminopropionic acid, DNP-alanine, DNP-capreomycidine, or DNP-serine (i.e. all the amino acids known to occur in capreomycin) in several different solvent systems. It was found that this unknown DNP-derivative had an R_F value of 0.38 in n-butyl alcohol saturated with a 1% aqueous ammonia solution.

An Analysis of the Hydrazides formed during Hydrazinolysis of Capreomycin Sulfate

The ethyl acetate extract containing the DNP-hydrazides of the DNP-amino acids formed in the preceding procedure, was evaporated to dryness. The residue obtained was heated with 6 N hydrochloric acid for 30 min on a steam bath. Using a rotary evaporator the aqueous hydrochloric acid was removed in vacuo, the residue was dissolved in 20 ml of a 5% sodium bicarbonate solution, and the solution was then extracted several times with ethyl acetate. These ethyl acetate extractions were discarded. Using dilute hydrochloric acid, the aqueous phase was acidified and then extracted with ethyl acetate which was then washed once

with water and dried over calcium chloride.

The DNP-derivatives in the dried ethyl acetate were compared on TLC with DNP-amino acid standards and it was found to contain DNP-alanine, bis-DNP-β-lysine, and bis-DNP-diaminopropionic acid.

Desureacapreomycin

Preparation of Desureacapreomycin

A sample of 0.5 g of crude capreomycin sulfate was dissolved in 12 ml concentrated hydrochloric acid. The solution was then heated rapidly on a hot plate. As soon as the solution started to boil 2 λ and 4 λ portions of the solution were removed by means of a micropipette and spotted separatedly on a silica gel plate. The solution was then returned to the hot plate and kept at a temperature just under the boiling point of the solution for 10 min. Two samples (2 λ and 4 λ) of this solution were then spotted on the same TLC plate.

A small sample of urea was then also introduced on the plate which was then developed with BAAAW. Visualization of spots was done first with p-dimethylaminobenzaldehyde and then with ninhydrin.

The samples taken from the hydrochloric acid solution as well as the urea standard gave a yellow spot with an R_F value of 0.58 with the p-dimethylaminobenzaldehyde reagent. It was also observed that the spots obtained for the 10 min hydrolysis were apparently of about the same size and intensity as those from the brief hydrolysis.

At the same time it was noted that the ninhydrin positive spot with an R_F value of 0.25 increased in size as the time of hydrolysis was prolonged. It therefore appeared that the brief hydrolysis was

sufficient to liberate the urea and that prolonged hydrolysis only led to increased hydrolysis of the remaining peptide.

Attempted Purification of Desureacapreomycin

A sample of 2 g capreomycin sulfate was partially converted into desureacapreomycin by boiling it briefly in 10 ml concentrated hydrochloric acid. The hydrochloric acid was then quickly removed under reduced pressure on a rotary evaporator and the residue thus obtained was dissolved in 8 ml of distilled water.

A column 105 cm x 2 cm was packed with CM-Sephadex-C-25 (H^+ form) and 2 ml of the desureacapreomycin solution was washed onto this column with 200 ml of distilled water. The column was further developed by gradient elution with dilute hydrochloric acid. For this gradient elution a reservoir flask was filled with one liter of a 0.6 N hydrochloric acid solution. The mixing flask contained 600 ml of distilled water at the beginning of the elution. A flow rate of 10 ml/15 min was maintained and 10 ml fractions were collected.

Each fraction was evaporated to dryness and the weight of the residue determined. From the data obtained a weight/volume curve was plotted and it was noted that the material came off the column as a single peak between fractions 13 and 23.

The leading and tailing fractions of the peak were analyzed on TLC with the solvent system BAAAW. No separation was observed since both the leading and tailing fractions contained strong ninhydrin positive spots with R_F values of 0.02, 0.08, and 0.65. Weaker ninhydrin spots were also observed. These spots had R_F values of 0.21 and 0.29.

The spot with an R_F value of 0.02 seems to be due to the presence of desureacapreomycin, and the spot with R_F 0.08 was due to unreacted capreomycin.

Hydrazinolysis of Desureacapreomycin

A sample of 0.65 g of crude desureacapreomycin, prepared as described above, was sealed with 10 ml of anhydrous hydrazine in a thick-walled glass tube and heated for 15 hr on a steam bath. The tube was then cooled, opened, and the contents dried *in vacuo* in a desiccator over concentrated sulfuric acid. The residue was dissolved in 20 ml of a 5% aqueous sodium bicarbonate solution. This mixture was then reacted, under constant stirring, with 0.9 ml of 2,4-dinitrofluorobenzene for 3 hr. Just enough 1 *N* hydrochloric acid was added to make the solution slightly acidic and it was then extracted with several portions of ethyl acetate.

The combined ethyl acetate extracts were then in turn extracted with 20 ml of a 4% sodium bicarbonate solution. The aqueous phase was washed several times with ethyl acetate and the ethyl acetate washings were discarded. After the aqueous phase was slightly acidified with hydrochloric acid, it was extracted again with ethyl acetate. The ethyl acetate phase from this extraction was dried over anhydrous sodium sulfate and was then filtered. Using a rotary evaporator the solvent was removed from the filtrate.

Most of the 2,4-dinitrophenol present in the residue was removed by sublimation under vacuum at 70° over a period of 12 hr. The remaining material was then subjected to a TLC analysis on silica gel using as solvent *n*-butyl alcohol saturated with a 1% aqueous ammonia solution.

One major yellow compound (R_F 0.38) and several minor compounds were observed.

None of these components compared with any one of the DNP-derivatives of the amino acids known to occur in capreomycin. The major compound also appeared to be the same as the compound which was found when capreomycin was analyzed for the identity of its C-terminal amino acid.

Degradation of Capreomycin

The Reaction of Capreomycin with Base

It was found that capreomycin sulfate in water had a maximum absorption at 265 mμ ($E^{1\%}_{1cm}$ 261) in the uv region of the spectrum. In aqueous 0.1 N, and aqueous 1.0 N sodium hydroxide the absorption shifted to 288 mμ ($E^{1\%}_{1cm}$ 101).

When the solution of capreomycin in 1 N sodium hydroxide was acidified, the uv absorption shifted back to 265 mμ. The intensity of this absorption did not differ from that of a solution of capreomycin sulfate in water if acidification took place soon after the solution in base was made up. It did, however, diminish when longer periods of exposure to the base were allowed.

A TLC analysis on a 1% solution of capreomycin in 1 N sodium hydroxide was carried out at various intervals. The decomposition products were visualized on the TLC plates with ninhydrin, Weber's reagent, and p-dimethylaminobenzaldehyde. The results of this analysis are given in Table 5.

It is interesting to note that none of the ninhydrin positive compounds released from capreomycin by this treatment corresponded in

Table 5. Hydrolysis of Capreomycin with 1N Sodium Hydroxide

Time of hydrolysis(days)	$E^{1\%}_{1cm}$(265 mμ)	R_F of components observed on TLC	Method of detection	Solvent System
1	210	0.08	Ninhydrin	BAAAW
		0.16	Ninhydrin	
2	200	0.05	Ninhydrin	
		0.10	Ninhydrin	BAAAW
		0.13	Ninhydrin	
		0.33	Ninhydrin	
		0.37	p-Dimethylamino-benzaldehyde	
4	142	0.05	Ninhydrin	
		0.10	Ninhydrin	
		0.13	Ninhydrin	BAAAW
		0.20	Weber + Ninhydrin	
		0.33	Ninhydrin	
		0.57	p-Dimethylaminoben-zaldehyde	
7	95	0.05	Ninhydrin	
		0.10	Ninhydrin	
		0.13	Ninhydrin	BAAAW
		0.20	Weber + Ninhydrin	
		0.33	Ninhydrin	
		0.57	p-Dimethylaminobenzal-dehyde	

R_F value to any of the amino acids known to occur in capreomycin. Urea was detected in the solution after two days using p-dimethylaminobenzaldehyde as spray reagent.

Hydrolysis of Capreomycin with a Saturated Barium Hydroxide Solution

A sample of 3.48 g of the free base of capreomycin was dissolved in 25 ml of a saturated aqueous barium hydroxide solution. The solution was heated on a steam bath for 12 hr and then allowed to stand at room temperature for a further 60 hr. Then the solution was filtered, and the barium ions in the filtrate were precipitated as barium carbonate with carbon dioxide. After the barium carbonate precipitate was removed by filtration, the filtrate was evaporated to dryness.

The residue was then reacted with 2.22 g of freshly precipitated cupric hydroxide in 5 ml of water for 12 hr. Insoluble material was then removed by filtration and the filtrate was subjected to gel chromatography on Sephadex G-25.

Sephadex G-25 was equilibrated with water and was packed to form a column 150 cm x 2 cm. The hydrolysate (complexed with copper) was eluted through this column with water at a flow rate of 1 ml/3 min. It was observed that the mixture was resolved on the column into a front running brown band followed by a bluish band.

The front and tail sections of both bands were analyzed on TLC with BAAAW. Three compounds which had R_F values of 0, 0.4, and 0.21 were detected with ninhydrin in the front section of the brown band. The tail fractions of this band had ninhydrin positive components with R_F values of 0, 0.04, 0.08, and 0.21. At least six components were found in all of the fractions of the blue band.

The brown band was collected separately and freeze dried. It was then rechromatographed over the same Sephadex G-25 column using water as solvent. No practical separation of the components in this band could be achieved in this way.

Hydrolysis of Capreomycin with 6 N Hydrochloric Acid at Room Temperature

A sample of 10 g of capreomycin sulfate was dissolved in 50 ml of a 6 N hydrochloric acid solution and the solution was kept at room temperature in the dark. Samples of this solution were analyzed after 4, 19, and 24 days on silica gel TLC plates with BAAAW. Ninhydrin positive compounds which had R_F values of 0, 0.03, 0.08, 0.15, 0.17, and 0.20 could be detected in all three cases. The spot which had an R_F value of 0.08 corresponded to the major component of capreomycin and it was found to diminish as the time of hydrolysis was increased. The compound which had an R_F value of 0.2 corresponded to β-lysine was the only one released during this hydrolysis.

After the hydrolysis was allowed to proceed for 24 days the solution was evaporated to dryness. A brownish residue was obtained.

Attempted Separation of the 6 N Hydrochloric Acid Hydrolysate on Sephadex G-25

Sephadex G-25 was equilibrated with a 3% formic acid solution and packed to form a column 120 cm x 2.5 cm.

A sample of 300 mg of the dried hydrolysate was dissolved in 2 ml of a 3% formic acid solution and was applied to the column. Further elution was also done with 3% formic acid. The flow rate was maintained at 10 ml/3 min and 5 ml fractions were collected.

A portion of each fraction was tested with ninhydrin for the

presence of peptides and it was found that the ninhdydrin positive material occurred in the fractions 58-86. Each of these fractions (58-86) were concentrated to about 1 ml under reduced pressure and analyzed on TLC with BAAAW.

It was observed that fractions 58-68 contained relatively pure material since only two components (R_F values of 0 and 0.03) were present. The fractions 69-75 had three components with R_F values of 0, 0.03, and 0.06. The rest of the fractions contained from 4 to 6 components with R_F values of 0, 0.03, 0.06, 0.15, 0.21 and 0.26.

On the basis of this TLC analysis, fractions 58-68, 69-75, and 76-86 were combined separately and called fractions I, II and III respectively.

The whole chromatographic procedure was repeated with 500 mg of the dried hydrolysate. Those fractions which were similar to fractions I, II and III were pooled separately and added to the corresponding fractions of the first chromatographic run. The total yields obtained in this way were 17.8 mg of fraction I, 129 mg of fraction II, and 537 mg of fraction III.

Attempted Purification of Fraction I by Paper Electrophoresis

All 17.8 mg of fraction I obtained from the Sephadex G-25 chromatographic separation of the 6 N hydrochloric acid hydrolysate was dissolved in 0.1 ml of water. By means of a micropipette 6 µl samples of this solution were applied to strips of Beckman no. 319328 electrophoresis paper. These papers were then treated with a pH 9.4 ammonium formate buffer and subjected to electrophoresis at a constant voltage of 105

volts for 3 hr. Sidestrips of these papers were then cut and reacted with ninhydrin. Three bands were detected at 0, 1.5 cm (-)* and 3.3 cm (-) from the origin. The two major components at 1.5 cm (-) and 3.3 cm (-) were eluted separately from the papers with an excess of 0.1 N formic acid and evaporated to dryness in vacuo. A TLC analysis of the two samples on silica gel with BAAAW showed that each band still contained two components with R_F values of 0 and 0.03.

Attempts to purify the small quantities of material left by crystallization from ethanol/water mixtures were unsuccessful.

Electrophoresis of Fraction II

The 129 mg of fraction II was dissolved in 0.7 ml of water, and 4 µl samples of this solution were then applied to Beckman no. 319328 electrophoresis paper strips. The papers were treated with a pH 9.4 ammonium formate buffer solution and were subjected to electrophoresis. After 14 hr at a constant voltage of 95 volts, the papers were dried. Sidestrips of these papers were treated with a ninhydrin solution which also contained 2,4,6-collidine, cupric chloride, and acetic acid (37). Three overlapping bands were observed at 8.6 cm (-), 10.1 cm (-), and 10.9 cm (-) from the origin.

The middle band at 10.1 cm (-) was extracted repeatedly with 0.1 N formic acid. The extract was then filtered and dried in vacuo to yield 41.6 mg of material which gave one spot with an R_F value of 0.03 with BAAAW.

To confirm the purity of this material it was subjected to TLC

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*The negative sign indicates that the compound has migrated towards the negatively charged electrode (cathode).

using various solvent systems. A solvent containing five volumes from the aqueous phase of n-butyl alcohol: water: acetic acid: pyridine: 5:8:1.5:1.5 and twenty volumes of BAAAW was found useful. This solvent was able to resolve the single spot observed with BAAAW into three well-defined spots with R_F values of 0.03, 0.1 and 0.14.

Further attempts to purify the mixture of peptides in the 6 N hydrochloric acid hydrolysate by means of electrophoresis were abandoned.

Gradient Elution of Fraction III on Dowex 50 W X-4

Dowex 50 W X-4 was converted into its protonated form as described previously (40). The resin was then suspended in 3 N hydrochloric acid and packed to form a column 120 cm x 2 cm.

All 537 mg of fraction III (obtained from the Sephadex G-25 chromatography of the 6 N hydrochloric acid hydrolysate) was dissolved in 5 ml of a 3 N hydrochloric acid solution and pipetted onto the column. Elution started with a 3 N hydrochloric acid solution and the strength of this solution was gradually increased by means of gradient elution.

Two, one-liter flasks were connected in series to the top of the column. The first flask contained 1 l of 6 N hydrochloric acid and the second flask contained 1 l of 3 N hydrochloric acid. The contents of the second flask was continuously stirred by means of a magnetic stirrer. Once flow started through the column the hydrochloric acid of the first flask siphoned into the second flask as the solution of this flask siphoned onto the column.

An automatic fraction collector was used to collect 10 ml frac-

tions of the eluate. Each fraction was concentrated in vacuo to about 1 ml and was then analyzed on silica gel thin layer with BAAAW. The first 23 fractions did not contain any ninhydrin positive material. Fractions 26-53 contained material which corresponded to the amino acids alanine, serine, diaminopropionic acid, capreomycidine, and β-lysine. The fractions beyond fraction 53 contained a complex mixture of peptides which were not resolved by the gradient elution.

Partial Hydrolysis of Capreomycin with Concentrated Hydrochloric Acid

A sample of 1 g of crude capreomycin was dissolved in 30 ml of concentrated hydrochloric acid in a pressure bottle. The bottle was stoppered tightly and then kept at 70^{o} for 6.5 hr on a water bath. By means of a rotary evaporator the aqueous solution was removed in vacuo. The residue was suspended in absolute ethyl alcohol and the ethyl alcohol was removed under vacuum. This procedure was repeated several times until the residue was thoroughly dired.

The peptides formed by the acid hydrolysis were converted into their ethyl esters by suspending the dried hydrolysate in absolute ethyl alcohol which has been saturated with dry hydrogen chloride gas. After keeping this solution protected from atmospheric moisture for two days, the ethyl alcohol and hydrogen chloride were removed in vacuo.

The residue thus obtained was then dissolved in a saturated sodium bicarbonate solution, 0.1 ml of 2,4-dinitrofluorobenzene was added, and the solution was agitated using a magnetic stirrer. After 3-5 hr the reaction mixture was extracted with ethyl acetate. The ethyl acetate extract was briefly washed with dilute hydrochloric acid and then with a dilute sodium bicarbonate solution. Subsequently, the ethyl

acetate was dried over anhydrous sodium sulfate for 24 hr, filtered, and the solvent removed under reduced pressure.

A TLC analysis of the yellow residue on silica gel with chloroform showed nine yellow spots with R_F values of 0, 0.04, 0.08, 0.14, 0.20, 0.31, 0.69, 0.77, and 0.88.

Chromatography of the DNP-Peptide Ethyl Esters on Silicic Acid

Activated silicic acid (Unisil, Clarkson Chemical Co. Inc.) was suspended in freshly distilled chloroform and the suspension then poured into a column and allowed to settle. A silicic acid column 44 cm x 1.5 cm was obtained.

All of the DNP-peptide-esters prepared as described above were suspended in 3 ml of chloroform and pipetted onto the column. The suspension was then carefully mixed with the top part of the silicic acid of the column by stirring with a glass rod. After allowing the top part of the column to settle again, the column was developed with chloroform, whereupon a total of six movable yellow bands were observed.

Each one of the yellow bands was eluted and collected separately. The fractions thus obtained were further tested for purity by means of TLC on silica gel with chloroform. If necessary the fractions were then further purified by preparative TLC on silica gel with chloroform. The major component of each fraction was then extracted from the silica gel with methanol and the purified material subjected to a mass spectroscopy analysis. The conditions used for the recording of each spectrum are given in Table 6.

Band I. Four different yellow spots were observed in this fraction; the major constituent had an R_F value of 0.43. The mass spectrum

of this compound was found to be identical to the mass spectrum of 2,4-dinitroaniline.

Band II. Four different components were also detected in this fraction by TLC. The major component had an R_F value of 0.45 and its mass spectrum is given in Table 7 under compound no. 1.

Band III. No further purification of this material was necessary. The mass spectrum obtained from it appears as compound no. 2 in Table 7.

Band IV. Two compounds were observed in this fraction, the major component had an R_F value of 0.22 and the minor component had an R_F value of 0.29. The major component was purified and its mass spectrum was taken (compound no. 3 Table 7).

Band V. In order to obtain a good separation of the components in this fraction, it was necessary to develop the TLC plate three times in the same direction with chloroform. Four different spots with R_F values of 0.07, 0.13, 0.20, and 0.24 were observed.

The major component (R_F 0.13) was purified and its mass spectrum recorded (compound no. 4, Table 7).

Band VI. It was also necessary in this case to develop the TLC plate three times in the same direction with chloroform to reveal five spots with the R_F values 0.04, 0.09, 0.16, 023, and 0.47.

The compound which had an R_F value of 0.16 was considered the major component of this mixture and was purified further as described above. Its mass spectrum appears in Table 7 (compound no. 5).

After the sixth band was eluted, the remaining material on the column did not appear to move to any appreciable extent. The column was then allowed to run dry and the silicic acid was carefully pushed

Table 6. Conditions Used for the Recording of the Mass Spectra Contained in Table 7

Compound	Temperature	Specific Amplitude	Region of Spectrum (m/e values)	Electron Current (μA)	Base Peak found at m/e =	Most Abundant Peak at m/e =
1	210°	2×10^{-12}	180-310	40	196	-
1	210°	2×10^{-13}	310	40	-	322
2	125°	1×10^{-11}	180-190	20	183	-
2	125°	1×10^{-12}	190-415	20	-	194
3	150°	2×10^{-12}	180	40	183	-
4	110°	2×10^{-12}	180	40	196	-
5	85°	2×10^{-12}	180	40	226	-
6	180°	2×10^{-12}	180-290	40	183	-
6	180°	2×10^{-13}	290	40	-	296
7	180°	2×10^{-12}	180-300	100	165	-
7	180°	2×10^{-13}	300	100	-	330
8	180°	1×10^{-12}	180	100	183	-
9	100°	2×10^{-12}	180	50	279	-
10	200°	2×10^{-13}	180	50	183	-
11	190°	2×10^{-12}	180-280	50	233	-
11	190°	2×10^{-13}	280	50	-	306
12	200°	2×10^{-12}	180-290	50	183	-
12	230°	2×10^{-13}	290	50	-	331
13	180°	5×10^{-12}	180	20	183	-
14	111°	5×10^{-12}	180-250	20	239	-
14	240°	5×10^{-13}	250	20	-	397

Table 7. Mass Spectra of the DNP-Ethyl Ester Derivatives Obtained from Partial Hydrolysis of Capreomycin*

Compound No.	Relative Intensities of Peaks at m/e = **											
	180	181	182	183	184	185	186	187	188	189	190	191
1	58	8		38	9					10	27	13
2	-	-	-	> 100	> 100	-	-	-	-	-	-	10
3	8	7	-	100	5	-	-	-	-	-	11	13
4	36	14	-	88	12	42	6	8	-	10	12	10
5	20	-	-	13	-	-	-	-	-	-	-	-
6	8	-	-	100	10	-	-	-	-	10	16	30
7***	-	-	-	5	-	-	-	-	-	-	11	5
8	17	14	-	100	20	7	6	8	10	18	22	36
9	9	-	-	5	-	-	-	-	-	-	-	-
10	-	-	-	100	17	-	-	-	-	-	-	-
11	20	44	7	11	-	6	-	18	-	7	7	7
12	22	6	-	100	15	-	-	-	-	30	45	41
13	5	6	-	100	9	-	-	-	-	-	12	12
14	14	18	20	32	10	24	14	18	-	16	-	14
	192	193	194	195	196	197	198	199	200	201	202	203
1	14	11	15	11	100	16	3	2	2	2	5	4
2	10	16	100	38	9	9	6	14	6	7	8	6
3	5	-	-	-	-	-	-	-	-	-	-	-
4	10	10	10	8	100	16	-	12	-	8	-	8
5	-	-	10	-	10	-	-	-	-	-	-	-
6	-	-	-	-	12	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	17	13	8	10	17	5	-	-	-	-	-	8
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	22	-	-	-	-	-	-	-
11	-	-	-	-	-	44	7	10	-	-	-	70
12	11	-	-	-	24	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	8	10	8	20	32	43	14	23	8	12	8	10

* Conditions under which these spectra were recorded are given in Table 6.

** Only peaks with relative intensities greater than 5% of either the base peak or the most abundant peak (depending on the region of the spectrum) are given.

*** The base peak for this compound was found at m/e 165.

Table 7 Continued

	204	205	206	207	208	209	210	211	212	213	214	215
1	6	7	14	-	-	-	15	-	-	-	-	-
2	6	11	8	8	6	7	14	9	6	21	6	8
3	-	19	6	-	42	-	-	-	-	-	-	-
4	-	9	-	-	6	-	28	7	-	6	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	20	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	7	28	13	12	25	8	5	5	-	6	5	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	10	8	7	-	-	-	-	-	-	12	-	-
12	6	78	23	14	7	-	-	-	-	-	-	-
13	-	18	-	-	20	-	-	-	-	-	-	-
14	-	10	-	13	7	13	14	26	-	18	-	10

	216	217	218	219	220	221	222	223	224	225	226	227
1	-	-	-	-	-	-	7	-	5	-	-	-
2	-	5	-	-	-	-	-	9	-	-	-	9
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	40	7
5	-	-	-	-	-	-	-	-	-	-	100	11
6	-	-	-	-	-	-	-	-	-	5	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	9	-	12	-	8	-	12	7	6	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	11	18	12	-	-	-	-	-	-	-	-	49
12	-	-	-	-	-	-	-	14	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	18
14	-	14	8	22	10	12	8	12	8	13	-	15

	228	229	231	233	234	235	236	237	238	239	240	241
1	-	-	-	-	-	-	36	-	-	-	-	-
2	10	9	7	-	-	-	7	6	-	7	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	6	-	-	-	14	10	-
5	-	-	-	-	-	-	-	-	-	-	7	-
6	-	-	-	-	-	16	14	6	-	8	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	12	6	6	8	8	-	9	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	5	-	-	7	-	-
11	-	-	-	100	10	-	-	-	-	-	-	-
12	-	-	-	-	5	48	8	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	-	10	8	6	-	12	7	22	12	100	26	28

Table 7 Continued

	243	245	246	247	249	250	251	252	253	254	255	256
1	-	-	-	-	-	16	-	7	-	-	-	-
2	-	-	-	-	-	-	-	6	-	-	13	21
3	-	-	-	-	-	-	16	-	-	-	-	-
4	-	-	-	-	-	-	-	-	8	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	10	-	4	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	3
8	-	-	-	9	6	8	21	6	6	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	5	-	-	-	-	-	-	-	-	-	-
11	35	-	6	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	46	5	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	12	17	-	10	8	-	19	22	20	12	16	14

	257	258	259	260	261	262	263	264	265	266	267	268
1	-	-	-	-	-	-	-	-	-	12	-	-
2	9	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	7
5	-	-	-	-	-	-	-	-	-	-	-	10
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	7	-	-	6	6	-	10	-
9	-	-	-	-	4	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	5	-	-	-
11	-	-	-	12	-	-	10	6	8	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	17	-
14	12	10	11	13	11	10	9	9	12	12	13	8

	269	270	271	272	273	274	275	276	277	278	279	280
1	-	-	-	-	-	-	-	-	-	-	-	-
2	6	7	11	8	11	-	9	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	8	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	8	-
9	-	-	-	-	-	-	-	-	-	-	100	25
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	7	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	10	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	13	8	11	8	13	13	10	10	8	34	12	8

Table 7 Continued

	281	282	283	284	285	286	287	288	289	290	291	292
1	-	15	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	12	7	-	-	-	-
3	9	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	8	-	-	-	-	-	-	-	-	-	-	-
9	5	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	20	-	-	40	34	-	60	32	-	20
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	32	12	8	16	8	8	11	12	29	13	10	8

	293	294	295	296	297	298	299	300	301	302	303	304
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	12	-	-	-	-	-	-	14	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	6	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	7	-	-	-	-	-
6	-	-	34	100	30	14	32	-	-	-	-	-
7	-	-	-	14	-	-	-	-	-	-	-	-
8	5	-	6	-	-	-	-	6	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	60	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	10	8	9	6	9	8	16	9	11	14	10	8

	305	306	307	308	309	310	311	312	313	314	315	316
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	12	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	40	-	30	-	60	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	5	-	-	9	-	6	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	100	26	15	-	-	-	-	-	20	-	-
12	-	-	20	-	-	-	-	-	-	-	80	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	6	11	8	5	10	5	8	6	16	7	13	9

Table 7 Continued

	317	321	322	323	324	325	326	327	329	330	331	332
1	-	-	100	21	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	9	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	100	50	46
8	-	-	-	-	-	4	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	60	40	80	40	-	-	-	100	40
13	-	-	-	-	-	-	-	-	-	-	-	-
14	7	8	27	14	6	8	-	9	-	-	17	7

	337	338	339	340	341	342	343	350	352	353	354	355
1	-	13	5	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	8	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	40	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	5	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	30	20	6	5	-	-	-	50	7	60	11	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	8	6	6	5	5	-	-	6	6	6

	356	357	361	363	367	368	369	374	375	376	381	382
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	7	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	60	-	-	-	-	-
7	-	-	-	-	-	-	-	76	48	91	-	-
8	-	-	-	-	-	8	5	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	18	36	-	-	-	-	-	-	-	-
12	-	-	-	30	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	5	5	-	-	6	9	7	-	-	-	10	11

Table 7 Continued

	383	394	395	396	397	398	399	400	412	413	414	415
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	15	8	-	8	-	7	-	24	7
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	9	24	20	v.s.	100	37	15	7	-	-	-	-

	538	552	553
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-
14	5	5	5

out by pressure supplied from a nitrogen cylinder.

The colored portion of the dried column was arbitrarily divided into two parts. Each of the two segments were extracted separately with methanol to obtain solution A from the front segment and solution B from the top segment of the column.

A TLC analysis of solution A on silica gel with ethyl acetate as solvent revealed eight components which had R_F values of 0, 0.10,

0.16, 0.36, 0.47, 0.59, 0.80, and 0.94. The major components (R_F 0 and R_F 0.59) were purified by preparative TLC on silica gel with ethyl acetate. They were then extracted from the plates with methanol and subjected to mass spectroscopy. The mass spectra of the components which had R_F values of 0, and 0.59 are given in Table 7 under compounds no. 6 and 7, respectively.

The mixture contained in solution B was also further purified by preparative TLC on silica gel with ethyl acetate. In order to obtain a good separation it was necessary to develop the plates three times in the same direction. The five bands nearest to the origin were extracted with methanol. The mass spectra of these compounds (in order of increasing R_F values) appears as compounds 8, 9, 10, 11 and 12 in Table 7.

The Mass Spectra of the DNP-Amino Acid Ethyl Ester Derivatives of the Amino Acids Found in Capreomycin

In order to interpret the mass spectra of the DNP-amino acid esters and DNP-peptide esters obtained as described above, it was necessary to obtain spectra of some known compounds to be used as reference.

The mass spectrum of DNP-alanyl methyl ester has been reported in the literature (38), and the mass spectrum of bis-DNP-β-lysyl-ethyl ester was given in Table 2.

Since capreomycidine does not yield a useful mass spectrum (27), and a useful mass spectrum has not been obtained for arginine (which also contains a guanidino group) (39), no attempt was made to obtain a spectrum of the DNP-derivative of this amino acid.

The ethyl ester of bis-DNP-diaminopropionic acid was prepared in the same way as the ester of bis-DNP-β-lysine. Its mass spectrum was recorded and is presented in Table 7 as compound 13.

Partial Hydrolysis of DNP-Capreomycin

A sample of 1.37 g DNP-capreomycin was hydrolyzed in 50 ml of 6 N hydrochloric acid in a pressure bottle for 6 hr on a steam bath. The hydrochloric acid was then removed *in vacuo*, and the residue was thoroughly dried in a desiccator over sulfuric acid. Subsequently, the sample was treated with dry ethyl alcohol which had been saturated with dry hydrogen chloride gas.

After the esterification was allowed to proceed for two weeks, the reaction mixture was filtered and the insoluble residue was extracted several times with a total of 100 ml of ethyl acetate. The material which remained insoluble was suspended in ethyl acetate and was then treated with an excess of acetyl chloride in the presence of sodium bicarbonate. During this process some of the yellow colored material dissolved in the ethyl acetate. The mixture was filtered, the filtrate was extracted with water, and the organic phase was dried over anhydrous sodium sulfate.

By means of a rotary evaporator the ethyl acetate was then removed under reduced pressure and the residue was dissolved in chloroform. The chloroform solution was eluted through a silicic acid column (40 cm x 1.5 cm) with chloroform. The first yellow band that came off the column was collected and its mass spectrum recorded (compound 14 in Table 7).

Hydrogenation of Capreomycin

Capreomycin sulfate (sample lot 566-532 AD-200, Eli Lilly and Company) was dried over concentrated sulfuric acid in a vacuum desiccator for 10 hr, and a sample of 4.515 g of the dry material then dissolved in about 20 ml of a 50% solution of distilled acetic acid in water.

In the meantime 5 g of a catalyst containing 10% platinum on carbon (Engelhard Industries, Inc.) was suspended in 300 ml of 50% acetic acid in water. The suspension containing the catalyst was stirred with hydrogen under a pressure of 1 atm for approximately 12 hr to equilibrate before the solution of capreomycin was introduced.

Hydrogenation under 1 atm and at room temperature was continued for a period of three weeks during which time 368 ml hydrogen (STP) was taken up by the capreomycin. After three weeks no further uptake of hydrogen was noticed and the reaction was terminated. The reaction mixture was filtered through a cake of Celite on a sintered glass funnel, and the solvent was removed *in vacuo* to give a fluffy white solid.

A sample of the reduced material was analyzed on silica gel HF_{254} with the solvent system BAAAW. Unlike capreomycin itself, the hydrogenated compound did not appear as a dark spot against a fluorescent background when the plate was viewed in short wavelength uv light. It did, however, react with ninhydrin to give a purple spot which corresponded in its R_F value to capreomycin itself. Also, it was possible to detect urea in the hydrogenated material by spraying the TLC plate with the *p*-dimethylaminobenzaldehyde reagent described before. Treatment of the reduced material with hydrochloric acid prior to analyses

on TLC did not increase the size of the urea spot.

Like capreomycin, the hydrogenated compound reacted with Weber's reagent but failed to give a copper complex when treated with copper (II) hydroxide.

Preparation and Properties of the Stoichiometric Sulfate of Hydrogenated Capreomycin

A 1 g sample of hydrogenated capreomycin was dissolved in 2.5 ml of water and pipetted onto an IR 45 (OH^-) column (46 cm x 2 cm). The column was developed with water until evaporation of eluate indicated that all of the material was washed through the column.

The eluate containing the hydrogenated capreomycin was concentrated *in vacuo* to approximately 5 ml. This solution was applied to an IR 45 ($SO_4^=$) column (37 cm x 2.5 cm) and eluted with 300 ml of water. Evaporation of the water by lyophilization gave 0.91 g of a white powder.

An nmr analysis on this material showed that the low field proton (1.9 τ) present in capreomycin was removed by the hydrogenation process.

It was also found that the position and intensity of the uv absorption of the hydrogenated material differed from that of capreomycin. Hydrogenated capreomycin had absorptions λ max at 256 mμ and λ max at 263 mμ in water and the intensities of these absorptions were $E^{1\%}_{1cm}$ and $E^{1\%}_{1cm}$ 74 respectively.

Biological Activity of Hydrogenated Capreomycin

The biological activity of hydrogenated capreomycin, measured by its ability to inhibit the growth of *B subtilis*,* was found to be 43%

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*This analysis was kindly performed by Dr. Paul D. Shaw and Mr. Rene' DuBos at the University of Illinois.

of the activity of capreomycin.

Determination of the N-Terminal Amino Acid of Reduced Capreomycin

A DNP-derivative of hydrogenated capreomycin was prepared in the same manner that the DNP-derivative of capreomycin was prepared. The DNP-derivative was then hydrolyzed in concentrated hydrochloric acid in a pressure bottle on a steam bath for 24 hr. The hydrochloric acid solution was evaporated under reduced pressure and the residue was suspended in water and extracted with ethyl acetate. Anhydrous sodium sulfate was used to dry the ethyl acetate extract. The extract was then filtered and the filtrate was evaporated to dryness.

Comparison by means of TLC on silica gel HF_{254} of the material thus obtained with bis-DNP-β-lysine revealed that they were the same. Several different solvent systems, including BAAAW, methyl alcohol, n-butyl alcohol, and ethyl acetate were used for this TLC comparison and the R_F values were found to be the same in all cases.

Hydrazinolysis of Hydrogenated Capreomycin

A sample of 0.39 g of hydrogenated capreomycin was sealed with 10 ml of anhydrous hydrazine in a thick-walled glass tube and heated for 15 hr on a steam bath. The tube was then cooled, opened, and the contents dried *in vacuo* in a desiccator over concentrated sulfuric acid. The residue was dissolved in 20 ml of a 5% aqueous sodium bicarbonate solution. This solution was reacted, under constant stirring, with 0.9 ml of 2,4-dinitrofluorobenzene for 3 hr. Just enough 1 N hydrochloric acid was added to make the solution slightly acidic and it was then extracted with several portions of ethyl acetate.

The combined ethyl acetate extracts were in turn extracted with

20 ml of a 4% sodium bicarbonate solution. The bicarbonate solution was washed several times with ethyl acetate and the organic washings was discarded. After the aqueous solution was acidified slightly with hydrochloric acid, it was extracted once more with ethyl acetate and this time the aqueous phase was discarded.

Anhydrous sodium sulfate was used to dry the ethyl acetate solution which was then filtered. The solvent was removed from the filtrate under reduced pressure on a rotary evaporator. Most of the 2,4-dinitrophenol present in the residue was removed by sublimation under vacuum at 70^{o}, the remaining material was subjected to a TLC analysis.

With n-butyl alcohol saturated with a 1% aqueous ammonia solution on silica gel HF_{254} a slightly reddish yellow spot with an R_F value of 0.38 was observed. Several minor components were also observed. The major compound (R_F 0.38) did not compare with any one of the DNP-derivatives of the amino acids known to occur in capreomycin. It also appeared to be the same as the compounds found when capreomycin and desureacapreomycin was analyzed for the identity of their C-terminal amino acids.

Hydrolysis of Hydrogenated Capreomycin with 6 N Hydrochloric Acid and Separation of the Hydrolysate by Ion Exchange Chromatography

Hydrogenated capreomycin (2.104 g) was hydrolyzed with 50 ml of 6 N hydrochloric acid in a pressure bottle on a steam bath for 36 hr. The hydrolysate was then evaporated to dryness, dissolved in water, and treated batchwise with IR 45 (OH^-) until the solution reached pH 6. The resin was then removed from the solution by filtration and the filtrate was concentrated under reduced pressure to a volume of about 10 ml.

This solution was pipetted onto a Dowex 50 (H^+) column (Dowex 50 W X-8, 100-200 mesh, Baker reagent 1930) which had the dimensions 5.5 m x 2.4 cm. The preparation and use of such a column for the separation of amino acids by gradient elution has been described previously (40). Gradient elution of the hydrolysate on this column with an increasing concentration of hydrochloric acid was done also as described by Floyd (40).

Fractions containing approximately 20 ml each were collected by means of an automatic fraction collector (GM Instrument Co., Inc. Model VE-2002-B24) at constant time intervals.

A small portion of every fifth fraction was tested for the presence of amino acids with ninhydrin. Once an amino acid peak has been located in this manner, the identity of the ninhydrin positive compound was determined by comparison with known amino acids on TLC. By analyzing leading and tail fractions from each peak the purity of the components was established.

Usually the solvent system BAAAW allowed the identification of most of the amino acids found in capreomycin. However, to be able to distinguish between alanine and serine, it was found necessary to use the solvent system CMA.

The fractions containing each amino acid were pooled separately, the solvent was removed in vacuo and the weight of each amino acid was determined. The data pertaining to this separation are given in Table 8.

Compounds known to be eluted between alanine and serine on a Dowex 50 (H^+) column includes glycine and glutamic acid. When these amino acids were compared to unknown I on silica gel with BAAAW and CMA

Table 8. Data Relating to the Separation of the Amino Acids in the Hydrolysate of Hydrogenated Capreomycin on Dowex 50 (H^+)

Fractions	Sample identified as	Sample weight (mg)	m. moles	Ratio
149-150	Serine	170	1.20	0.79
168-184	Unknown I*	90	-	-
187-198	Alanine	419	3.348	2.22
226-232	Unknown II*	36	-	-
272-279	Diaminopropionic Acid	528	2.98	1.98
291-302	β-Lysine	384	1.75	1.16
363-378	Capreomycidine	368	1.50	1.00

*The unknown compounds were not peptides since further hydrolysis with 6 N hydrochloric acid did not change them. Unknown II was found to be Weber positive.

they did not correspond.

Determination of the Optical Rotations of the Amino Acids isolated from the Hydrolysate of Hydrogenated Capreomycin

The amino acids obtained from hydrogenated capreomycin were further purified by treatment with activated carbon. Each sample was separately dissolved in a small volume of water, activated carbon (Darco G 60) was added, and the solution was heated to boiling. The solutions were then filtered through Celite, and the water was removed by means of lyophilization.

A sample of each amino acid obtained in this way was dissolved in 5 N hydrochloric acid and its optical rotation determined using a Bellingham and Stanley polarimeter. The $[\alpha]_D$ values obtained are

alanine +18.7°; diaminopropionic acid +12.5°; and β-lysine +19.2°.

The Reaction of Capreomycin with Cupric Ions

In a typical reaction 1 g of capreomycin sulfate was dissolved in 70 ml of water; 2 g of copper carbonate and 10 g of Amberlite IR-45 (OH^-) were added and the mixture was stirred for 16 hr. The resin and unreacted copper carbonate was removed by filtration, and the reddish-purple filtrate was dried by means of lyophilization to give 0.601 g of a purple solid.

A sample of 0.53 g of this preparation was dissolved in 0.5 ml of water and pipetted onto a Sephadex G-15 column (100 cm x 1 cm) which had been equilibrated and packed in water. The colored material was eluted at a flow rate of 2 ml/min. Three colored bands were observed to move down the column, one of which apparently was a minor component and which moved only very slowly.

The first two bands were collected separately and dried by means of lyophilization. Band I (the first to be eluted) weighed 190 mg and Band II weighed 80 mg. A TLC analysis on silica gel HF_{254} with BAAAW revealed that Band I contained only one component (R_F 0.20) while Band II showed two spots (R_F 0.20 and R_F 0.25).

Each band was also tested with a barium chloride solution, and it was found that Band I contained sulfate as the anion, while Band II contained the carbonate anion.

Both of these compounds were subjected to electrophoresis in a 0.3% sodium borate buffer (pH 8.5) at 150 v for 1.5 hr on Beckman no. 320046 paper strips. After development, the papers were dried in air and viewed under a short wavelength uv light. Three different components

could be observed in both cases, all of which migrated towards the cathode. The distances these compounds moved from the point of application was 2.7 cm, 1.8 cm, and 0.7 cm, respectively. The compound at 0.7 cm had a bluish color and it reacted with ninhydrin, as did the other two components.

Attempted Separation of Capreomycin Complexed with Copper from Uncomplexed Capreomycin

A sample of 10 g of the mixture of capreomycin-copper-complexes was dissolved in water and slowly washed through an Amberlite IR-400 resin in the chloride form. The eluate containing the colored material was lyophilized and gave 8.5 g of a purple solid. This material did not contain sulfate or carbonate as no precipitate formed with barium chloride; it did, however, give a positive test for chloride.

Chelex 100 resin, complexed with cupric ions, was suspended in water and packed in a glass column to form a green-blue column 41 cm x 2.5 cm. One gram of the chloride form of the capreomycin-copper-complex was dissolved in 2 ml of water and was applied to the Chelex column.

Elution with a large volume of water was not able to move the capreomycin through the column. Only some uncomplexed copper chloride was found in the eluate.

Chromatography of the Capreomycin-Copper-Complex (Chloride Form) on Sephadex G-15

A sample of 15 g of the capreomycin-copper-complex which had been converted into its chloride form was dissolved in 2 ml of water and pipetted onto a 72 cm x 2.5 cm Sephadex G-15 column. The complex was eluted with water at a flow rate of 1 ml/10 min. No clear-cut separation of bands could be observed, but it was noted that the leading

part of the colored band was more red in color while the trailing part was somewhat bluish.

It was estimated where the front reddish part of the band ended and this was collected separately from the trailing section of the colored band. Each fraction was dried by means of lyophilization. The front part of the band yielded 178 mg, and the last part gave 93 mg.

The Sephadex G-15 column was washed thoroughly with water, and the 178 mg from the front portion of the colored band was dissolved in 0.5 ml of water and chromatographed again on the same column. This time the band that moved down the column appeared to be homogeneous in color. Nevertheless, only the front four-fifths (approximately) of this band was collected and freeze-dried to yield 79 mg of the complex.

Thin layer chromatography with BAAAW on silica gel and electrophoresis in a 0.3% sodium borate buffer (pH 8.5) at 450 v for 45 min indicated that this material was pure. A uv spectrum of this material was taken and it was found that it absorbed at the same wavelength and with the same intensity as uncomplexed capreomycin sulfate.

All attempts to crystallize the copper complex of capreomycin failed.

Estimation of the Molecular Weight of the Capreomycin-Copper-Complex (Chloride Form)

The amount of cupric ions in a sample of the capreomycin-copper-complex (which was purified by chromatography on Sephadex G-15) was determined by titration with a 0.0050 M solution of disodium EDTA (41).

A sample of 24.2 mg of the capreomycin complex was dissolved in 10 ml of water, and 50 ml of a 50% aqueous ethanol solution was added.

Also 5 ml of a pH 5 acetate buffer and five drops of Pan* indicator was added to the solution. It was then titrated with a 0.0050 M disodium EDTA solution until the color of the solution changed from a deep red-violet to a greenish-yellow. It was necessary to add 4.30 ml of the EDTA solution before the end point was reached. This was equivalent to $4.30 \times 0.0050 = 2.15 \times 10^{-5}$ moles of copper.

If capreomycin formed a 1:1 complex with the cupric ions the titration data would indicate that the complex had a molecular weight of 24.2/0.02150 = 1125. Furthermore, if the weight of cupric chloride is subtracted from this value, an approximate molecular weight of 990 is obtained for capreomycin itself.

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*Pan = 1-(2 pyridylazo)-2-naphthol.

CHAPTER III

DISCUSSION OF RESULTS

Purification and Analytical Results

Before any separation attempts could be carried out, it was necessary to develop a method which could be used to distinguish between the various components of the capreomycin mixture. Thin layer chromatography on silica gel HF_{254} plates with BAAAW separated commercial capreomycin into four components. Two of these components could only be observed when relatively large concentrations of the material were applied to the plates.

Potentiometric titration of the capreomycins indicated that capreomycins IA and IB had larger molecular weights than capreomycins IIA and IIB. Gel filtration using Sephadex gels are able to separate such compounds with different molecular sizes on the basis that smaller molecules penetrate the pores in the gel more readily than larger molecules and are, thus, eluted less readily. It was, however, found that gel filtration using either Sephadex G-10 or Sephadex G-15 was not able to separate the capreomycin mixture.

A material that combines the properties of a gel and an ion exchange resin is DEAE-Sephadex A-25. A mixture of the free bases of capreomycin was eluted through such a DEAE-Sephadex A-25 (CH_3COO^-) column with a pyridinium acetate solution, and the fractions obtained were analyzed by TLC. Those fractions which contained only the major component were combined and were then further purified from pyridinium

acetate by gel chromatography over Sephadex G-10.

This purified material was hydrolyzed in acid and the hydrolysate was analyzed with an amino acid analyzer. The values obtained shows that serine, alanine, β-lysine, diaminopropionic acid, capreomycidine, and ammonia occurred in the ratio of 0.24: 0.87: 2.24: 2.64: 1.00: 1.95 in this material.

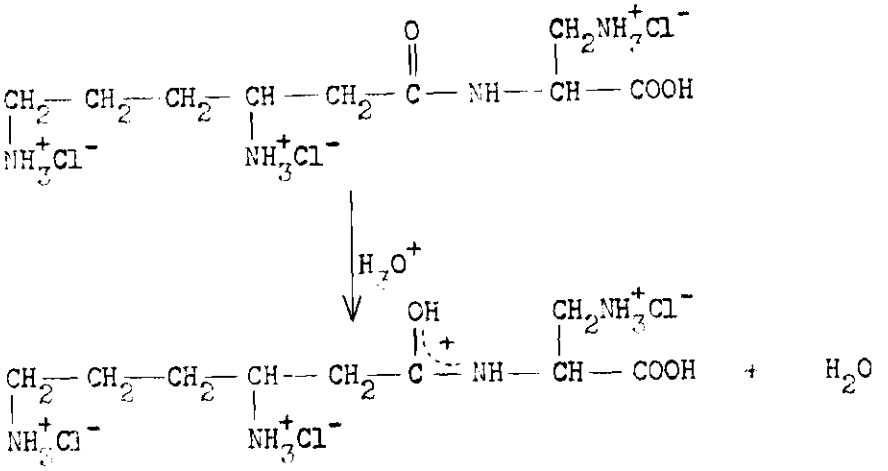
It is interesting to note that higher values were obtained for β-lysine, diaminopropionic acid, and ammonia than were found previously (Table 2). Higher values for β-lysine and ammonia have also been observed for viomycin which was purified by gel chromatography (26). In the case of viomycin, it was speculated that the high value of β-lysine might be due to the fact that it is a very hygroscopic compound. It would, therefore, have been difficult to obtain accurate weights on standards of this amino acid. This explanation did not account for the high value of ammonia in viomycin. In the case of capreomycin such reasoning also does not explain the high values obtained for ammonia and diaminopropionic acid.

The discrepancy in the results of the amino acid analyses of capreomycin might be due to differences in the conditions of hydrolysis. Bodansky et al. (20) has found recently that in the case of stendomycin, an antibiotic related to capreomycin, the correct amino acid ratios were obtained only if hydrolysis with constant boiling hydrochloric acid at 110° was carried out for about 90 hr. In this case the resistance of the peptide to acid hydrolysis was attributed to the occurrence of amino acids with bulky non-polar side chains next to each other.

In the case of the data obtained on capreomycin it can be noted

that the difference between these recent results and previous amino acid analyses corresponds roughly to 1 equivalent more of each of β-lysine and diaminopropionic acid. The dipeptide β-lysyl-diaminopropionic acid has been found in the hydrolysate of capreomycin IB (8), and it can be imagined that this peptide will be resistant toward acid hydrolysis.

Scheme I



The attack of the amide bond by a proton (Scheme I), a necessary step for hydrolysis, will be sterically hindered by the bulky β-lysyl side chain. It can also be imagined that further protonation of a molecule that has already been protonated at three sites would be a difficult feat from an electrostatic standpoint. Resistance of this peptide towards acid hydrolyses could, therefore, explain the lower values previously obtained for β-lysine and diaminopropionic acid.

Further examination of the recent amino acid analysis also revealed that although many of the amino acids occurred in nonstoichiometric ratios,

the sum of all the amino acids was 6.99 (i.e. 7.00) moles. This indicated that the purified capreomycin, although it gave a single spot on TLC, was still a mixture of components. The data also indicated that the dominant component of this mixture (capreomycin IB) contained alanine, β-lysine, diaminopropionic acid, capreomycidine, and ammonia in the ratio of 1:2:3:1:2.

From the presence of serine in the hydrolysate, it was concluded that capreomycin IA was also present in the mixture. The values of β-lysine and diaminopropionic acid suggested that one of the diaminopropionic acid units of capreomycin IB was replaced by a β-lysine unit in capreomycin IA. Therefore, the ratio serine: β-lysine: diaminopropionic acid: capreomycidine: ammonia:: 1:3:2:1:2 can be suggested for capreomycin IA.

Using these ratios for capreomycins IA and IB, and by using the value obtained for serine as an indication of the amount of capreomycin IA present in the mixture, the mole ratio of the amino acids in the mixture was calculated. The results are summarized in Table 9.

All attempts to separate capreomycins IA and IB failed, and therefore, further degradative studies were carried out on a mixture of the capreomycins. This mixture contained approximately 67% of capreomycin IB, 25% of capreomycin IA, 6% of capreomycin IIB and 3% of capreomycin IIA (8).

End-Group Analyses

N-Terminal Amino Acid Analyses

A DNP-capreomycin derivative was prepared by reacting capreomycin

Table 9. Observed and Calculated Ratios of Amino Acids in a Mixture of Capreomycins IA and IB

	Ser	Ala	β-Lys	Dapa	Capr	Ammonia
Capreomycin IA	0.24	0	0.7	0.48	0.24	0.48
Calculated						
Capreomycin IB	0	0.76	1.52	2.28	0.76*	1.52
Total	0.24	0.76	2.24	2.76	1.00	2.0
Found	0.24	0.87	2.24	2.64	1.00	1.95

*This value was calculated from the concept that both peptides contain one mole of capreomycidine.

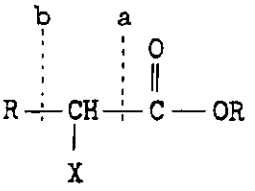
with 2,4-dinitrofluorobenzene in a basic solution. The DNP-capreomycin was hydrolyzed in 12 N hydrochloric acid. The precipitate which formed during the reaction was collected by filtration and the filtrate was extracted with ethyl acetate. TLC analysis of the precipitate and the ethyl acetate extract showed that both were identical with bis-DNP-β-lysine. This indicated that both amino groups of a β-lysine unit are not bonded in capreomycin.

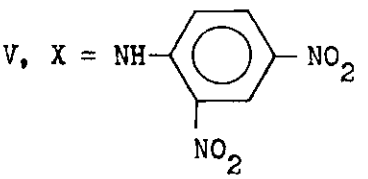
In viomycin the free amino groups of β-lysine gave rise to pKa values of 8.2 and 10.3 (23,30). Similar pKa values (8.2 and $>$ 10) in capreomycin (8) confirmed the finding that both amino groups of a β-lysine unit are free in capreomycin. Herr and Redstone reported (8) that capreomycin contained only one mole of β-lysine. They also found that one of the nitrogens of a β-lysine was bonded to alanine since they were able to isolate the peptide alanyl-β-lysine from a partial acid

hydrolysate of capreomycin. Therefore, this β-lysine could not account for the formation of bis-DNP-β-lysine.

It was, therefore, necessary to present additional proof that the DNP-derivative obtained by acid hydrolysis from DNP-capreomycin was actually bis-DNP-β-lysine. This was done by taking a mass spectrum of the ethyl ester of this DNP-derivative. The mass spectra of some DNP-amino acid methyl esters have been reported (38). The characteristic peaks in most of these spectra are due to the breaking of bond a (formula V). This is in accordance with the fragmentation observed for amines where the bond one removed from nitrogen is cleaved preferentially (42).

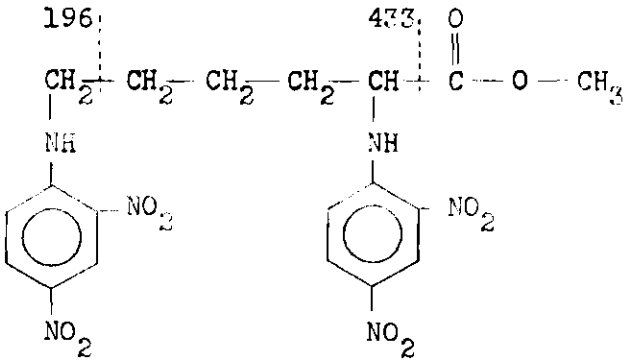
The same type of fragmentation has been observed (39) for the ethyl esters of amino acids (formula VI). Cleavage of bond b in the DNP-amino acid esters apparently did not take place readily unless the β-carbon was highly substituted or contained a heteroatom. Cleavage of bond b (formula VI) in amino acid esters gives a much smaller peak than the one obtained from cleavage of bond a because in the resulting ion the positive charge is destabilized by the neighboring carbethoxy group (43).





VI, X = NH_2

Amine type cleavages involving both nitrogens of bis-DNP-lysine methyl ester (formula VII) were observed (peaks at m/e 196 and m/e 433). The methyl ester of bis-DNP-lysine also underwent fragmentation through the loss of 2,4-dinitroaniline and one hydrogen (184 mass units) to give a strong peak at m/e 308. The loss of 2,4 dinitroaniline from the amine peak at m/e 433 gave rise to a strong peak at m/e 250 (38). Similar fragmentations involving the loss of ammonia have been observed for the ethyl ester of lysine (39).



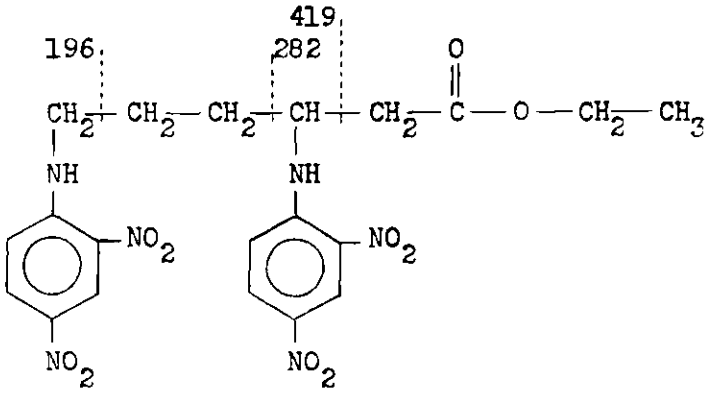
VII

Bis-DNP-β-lysine would be expected to give a peak at m/e 196 from an amine type fragmentation at the ε-DNP group in the molecule. Amine type fragmentations on both sides of the β-carbon could also be expected since destabilization of the positive charge in the ion containing the carbethoxy group was expected to be less severe because the carbonyl group is removed one methylene group from the center carrying the charge. Peaks at m/e 419 and m/e 282 would result from such a fragmentation.

Fragmentation of bis-DNP-β-lysine ethyl ester through the loss of 184 mass units (2,4-dinitroaniline + hydrogen) from the molecular ion

could be expected by comparison with bis-DNP-lysine methyl ester. Similarly a loss of 183 mass units (2,4-dinitroaniline) from the amine peak containing both DNP-substituents could be expected to lead to a peak at m/e 236.

All these peaks expected for bis-DNP-β-lysine ethyl ester (formula VIII) was found to be present in the spectrum (compound 1, Table 4) of the ethyl ester of the DNP-derivative isolated from the acid hydrolysate of DNP-capreomycin.



VIII

This mass spectrum therefore established that a β-lysine unit with both amino groups free occurs in capreomycin. These amino groups accounted only for the pKa values 8.2 and 10 observed for capreomycin, leaving a basic group with a pKa value of 6.3 unaccounted for. Of all the amino groups of the amino acids known to occur in capreomycin, the pKa value of 6.6 reported (44) for the α-amino group of diaminopropionic acid most closely approaches this value. It appears that the pKa value of the amino group of an amino acid is lowered somewhat when this amino group appears as a free amino group in a peptide (Tables 4-11 p. 486 in

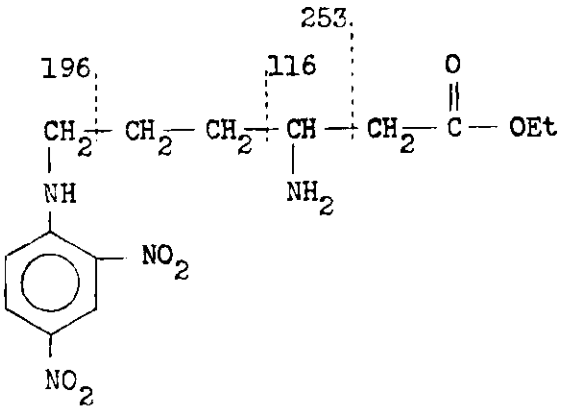
reference 44). Therefore, it would be expected that the pKa value for the α-amino group of a diaminopropionic acid group in the capreomycine peptide will be lower than the value reported for the free acid and will thus be more in line with the pKa value of 6.3 reported for capreomycin. If the α-amino group of diaminopropionic acid is indeed free in the intact capreomycine molecule it would be expected that hydrolysis of DNP-capreomycin would release α-mono-DNP-diaminopropionic acid. An attempt was, therefore, made to identify this compound in the acid hydrolysate of DNP-capreomycin.

After bis-DNP-β-lysine was removed from the acid hydrolysate of DNP-capreomycin through filtration and extration with ethyl acetate, a yellow color remained in the aqueous solution indicating the presence of one or more DNP-derivatives. This material was compared with 2,4-dinitrophenol, DNP-alanine, DNP-serine, α-mono-DNP-capreomycidine, α- and β-mono-DNP-diaminopropionic acid, bis-DNP-β-lysine, and bis-DNP-diaminopropionic acid on TLC. None of these DNP-derivatives corresponded to the DNP-derivatives remaining in the acid hydrolysate.

An attempt was therefore made to isolate some of these DNP-derivatives in the acid hydrolysate by chromatography on preparative paper. The major yellow band obtained in this way was extracted from the paper. It was then treated with anhydrous ethanol saturated with anhydrous hydrogen chloride and was further purified by preparative TLC. Four different yellow bands were observed. Though mass spectra were taken of the two major yellow bands thus obtained (compounds 2 and 3, Table 4), it was not possible to interpret these spectra.

In an additional attempt to isolate DNP-derivatives from the acid

hydrolysate of DNP-capreomycin the hydrolysate was esterified and was then subjected to preparative TLC. A mass spectrum of the main yellow band thus obtained was taken (compound 4, Table 4). Peaks at m/e 196, m/e 116, and m/e 253 in this spectrum corresponded to the peaks expected from the amine type fragmentation in ε-mono-DNP-β-lysine ethyl ester (formula IX).



IX

It was also expected from the fragmentation patterns of the esters of bis-DNP-lysine and bis-DNP-β-lysine that loss of 184 mass units (2,4-dinitroaniline plus one hydrogen) or 18 mass units (ammonia plus one hydrogen) from the molecular ion would take place. Such fragmentation in ε-mono-DNP-β-lysine ethyl ester was expected to give rise to a peak at m/e 156 or at m/e 322. A strong peak at m/e 156 was indeed observed, indicating that elimination of 2,4-dinitroaniline and a hydrogen was favored over elimination of ammonia and a hydrogen. A fragment with m/e 322 was not observed but a peak at m/e 323 indicated that loss of ammonia, unlike the loss of 2,4-dinitroaniline, was not accompanied by

a loss of one hydrogen.

A N-terminal amino acid analysis was also carried out on hydrogenated capreomycin using the same method as for capreomycin. The DNP-derivative thus obtained was shown to be identical to bis-DNP-β-lysine in all TLC systems used. From this it could be concluded that hydrogenation of capreomycin did not have effect on the N-terminal group of the peptide.

C-Terminal Amino Acid Analysis on Capreomycin

Capreomycin was treated with anhydrous hydrazine according to the procedure developed by Akabori et al (45). The hydrazine was removed in vacuo, and the residue was reacted with 2,4-dinitrofluorobenzene in a slightly basic solution. After acidification, the solution was extracted with ethyl acetate. The DNP-derivative of the C-terminal amino acid was isolated from the DNP-amino acid hydrazides by extracting it from the ethyl acetate solution with a sodium bicarbonate solution. When this aqueous sodium bicarbonate solution was acidified, it was possible to extract all of a yellow DNP-derivative into an ethyl acetate solution. This behaviour indicated that the yellow compound was indeed a DNP-derivative of an acid. If any one of the amino acids known to occur in capreomycin is C-terminal, this DNP-derivative could have been only DNP-alanine, bis-DNP-β-lysine, bis-DNP-diaminopropionic acid, or DNP-capreomycidine. A TLC analysis showed that the DNP-derivative obtained did not correspond to any of these DNP-amino acids. It could, therefore, be concluded that none of the known amino acids in capreomycin is C-terminal.

The DNP-amino acid hydrazides which represent the non-terminal

amino acids were also investigated. These hydrazides were hydrolyzed to the corresponding DNP-amino acids. Using TLC, DNP-alanine, bis-DNP-β-lysine, and bis-DNP-diaminopropionic acid were identified. Since capreomycin contains more than one molecule each of β-lysine and diaminopropionic acid, it was not possible, on this evidence alone, to rule out the possibility that either one could have been C-terminal. The fact that DNP-alanine was released by hydrolysis of the DNP-hydrazides proved unambiguously that alanine was not C-terminal in capreomycin.

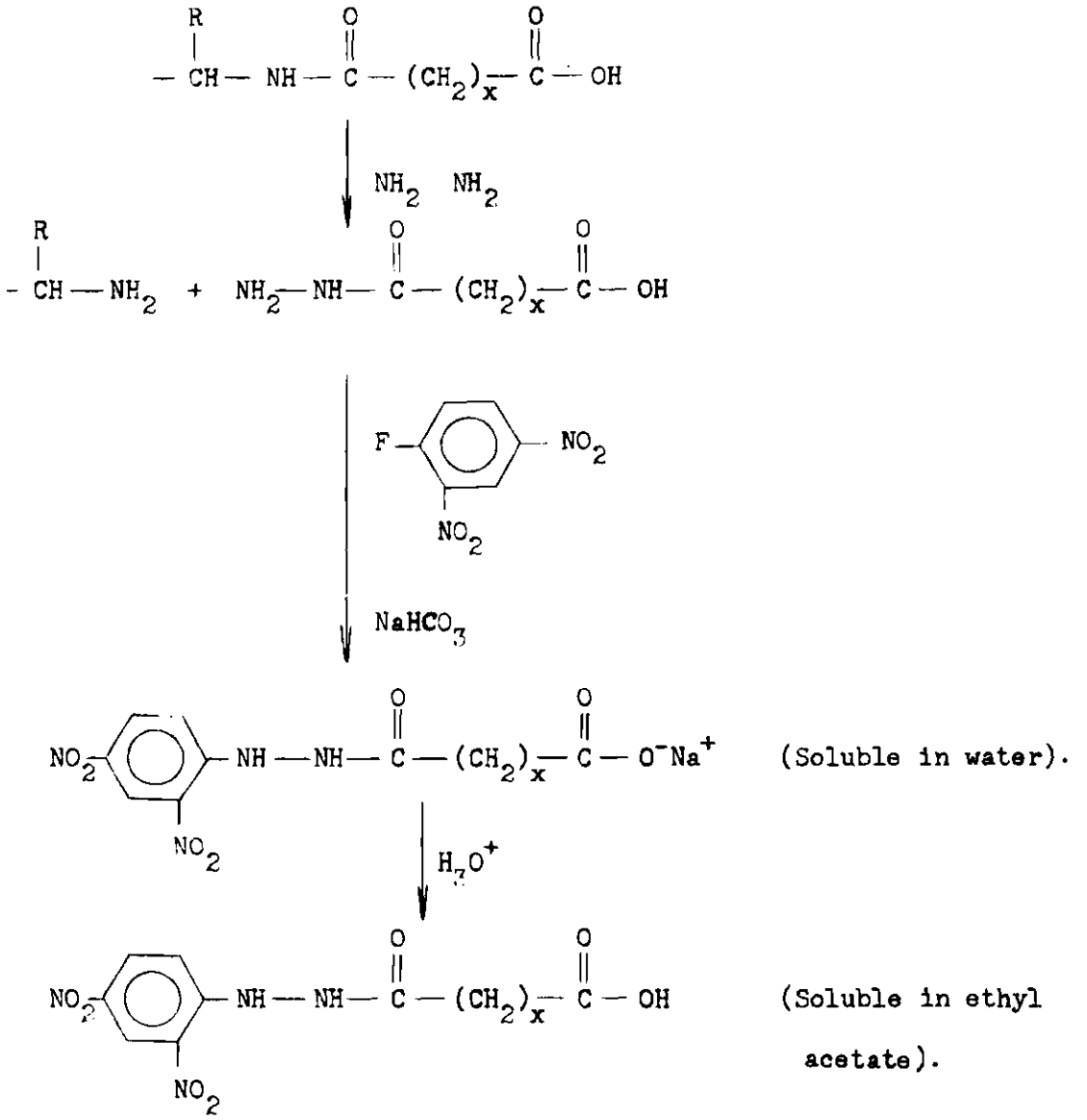
It was interesting to note that DNP-capreomycidine was not released by the hydrolysis of the DNP-hydrazides. This observation can be explained by the fact that the quanidino group present in this molecule would make it more soluble in aqueous acidic solutions than in organic solvents. The DNP-capreomycidine hydrazide was therefore not extracted into the first ethyl acetate extract with the other DNP-hydrazides, but remained in the aqueous solution.

Furthermore, it is also possible that the DNP-derivative isolated does not originate from a C-terminal amino acid but from a dicarboxylic acid which had only one carboxylic acid group free in the intact molecule. Hydrazinolysis would then have converted such an acid into a mono-hydrazide derivative which would have behaved similarly to a C-terminal amino acid as shown in Scheme II.

C-Terminal Amino Acid Analysis on Desureacapreomycin

It has been reported that urea was released from capreomycin upon mild acid hydrolysis (15) to yield desureacapreomycin. Brief treatment of capreomycin with concentrated hydrochloric acid also led to the

Scheme II


NH2 NH2
NaHCO3
H3O+
(Soluble in water).
(Soluble in ethyl acetate).

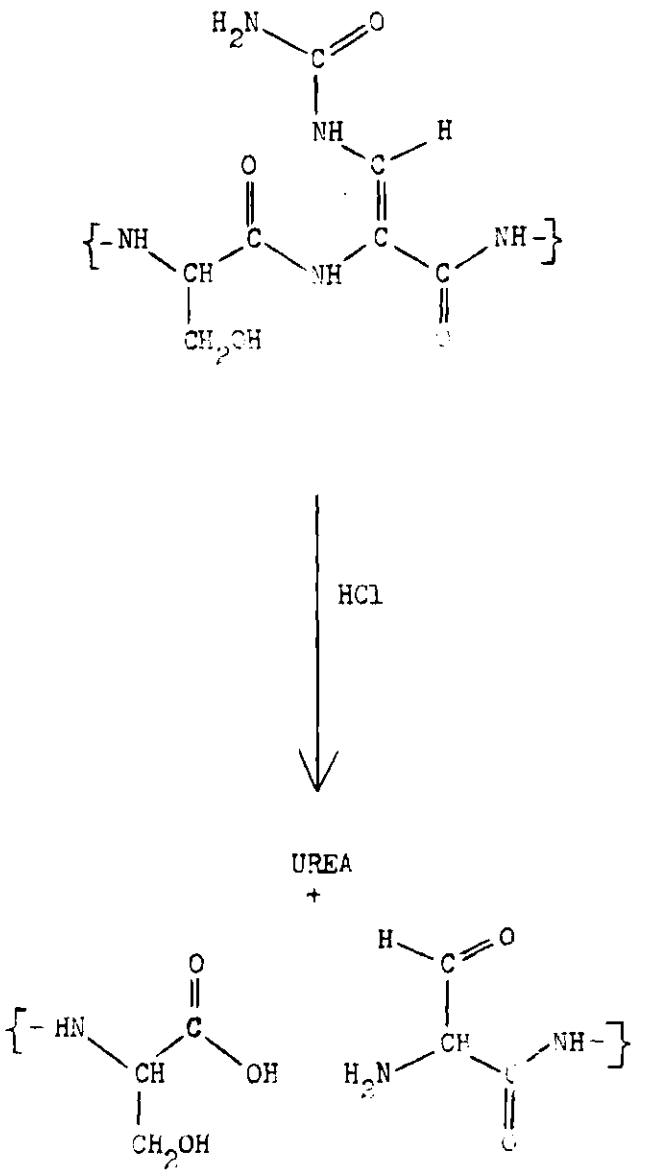
release of urea. Attempts to obtain desureacapreomycin pure by means of gradient elution of CM-Sephadex C-25 were unsuccessful. Thus, a C-terminal amino acid analysis was carried out on a crude preparation of desureacapreomycin using the same procedure as for capreomycin.

The DNP-derivative obtained in this way again did not correspond on TLC to the DNP-derivatives of the amino acids occurring in capreomycin. Furthermore, this DNP-derivative appeared to be the same as the one obtained from the C-terminal analysis of capreomycin itself. Both compounds had an R_F value of 0.38 on silica gel with n-butyl alcohol saturated with a 1% ammonium hydroxide solution. This analysis showed that no free carboxylic acid group became free when urea was released from capreomycin. It can thus be concluded that the urea was not released from an ureide in capreomycin.

Mild acid hydrolysis of viomycin led to the release of urea. The desureaviomycin produced in this way contained serine as a C-terminal amino acid, whereas viomycin did not possess a C-terminal amino acid. It was proposed by Bycroft et al (25) that hydrolysis of the chromophore could have led to the formation of a C-terminal amino acid in desureaviomycin as outlined in Scheme III.

If the chromophores of viomycin and capreomycin are the same, it would be expected that desureacapreomycin would also have a C-terminal amino acid, generated by a mechanism similar to the one outlined in Scheme III. The fact that a C-terminal amino acid was not found in desureacapreomycin indicates that either the chromophore proposed (25) or the suggested mechanism by which urea was released from the chromophore (Scheme III) is in error.

Scheme III



It was also necessary to consider the possibility that the proposed chromophore (formula III) occurred as an N-terminal group in capreomycin since this could explain the observation that hydrolysis of this group did not create a C-terminal amino acid. Dehydroamino

acid derivatives of the type proposed for the chromophore are, however, only stable in form of their N-acyl derivatives (20). This fact, together with the observation that alanine is not N-terminal in hydrogenated capreomycin, excluded the possibility that the dehydroamino acid derivative was N-terminal.

C-Terminal Amino Acid Analysis on Hydrogenated Capreomycin

A C-terminal amino acid analysis was carried out on hydrogenated capreomycin using the same procedure as for capreomycin and desureacapreomycin. The DNP-derivative obtained appeared to be the same compound as that obtained from C-terminal amino acid analyses on capreomycin and desureacapreomycin. It is of interest to note that a C-terminal alanine was not found for hydrogenated capreomycin. This observation excluded the possibility that the dehydroamino acid derivative (formula III) proposed as the chromophore occurs as the C-terminal group in capreomycin.

Partial Degradation of Capreomycin

The course of the hydrolyses of capreomycin in 6 N hydrochloric acid and 1 N sodium hydroxide solutions at room temperature was followed by means of TLC using the solvent system BAAAW. The only amino acid found to be liberated in noticeable quantities was β-lysine which was observed in the acid hydrolysate after four days. Both methods of hydrolyses released urea, and a complex mixture of peptides was formed in both cases.

In the first attempt to obtain some of these peptides pure for analysis capreomycin was hydrolyzed with a barium hydroxide solution.

Barium hydroxide was preferred because it is easy to remove the barium ions after the hydrolysis by means of precipitation as barium carbonate. The mixture of peptides was then treated with freshly precipitated copper (II) hydroxide. The resultant mixture of copper-peptide-complexes was chromatographed over Sephadex G-25 and each of the fractions obtained was tested for purity by means of TLC. This analysis showed that none of the fractions contained pure material.

Gel chromatography on Sephadex G-25 with a dilute formic acid solution was used in an effort to separate the mixture of peptides obtained from the room temperature 6 N hydrochloric acid hydrolysis. No clear-cut separation was observed. Those fractions which showed two spots on TLC were combined and was called fraction I. Also, those fractions containing three spots and those containing more than three spots were pooled separately and were called fractions II and III respectively.

Electrophoresis did not separate the components in fraction I. On the other hand, electrophoresis of fraction II followed by extraction of the paper strips yielded some material that showed only one spot on TLC with BAAAW. By using a different solvent system it was possible to show that this material actually contained three different components. Electrophoresis was not successful for the separation of these peptides and a different approach was therefore tried to separate the peptide mixture in fraction III.

Gradient elution, using increasing concentration of hydrochloric acid on Dowex 50 W X-4, was carried out on fraction III. The concentration of acid required to elute the peptides from the resin was apparently

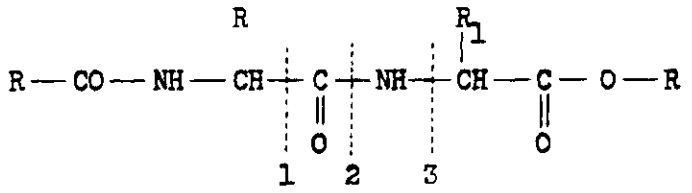
high enough to cause extensive hydrolysis of the peptides since most of the fractions containing peptides also contained relatively high concentration of free amino acids; hence this method was also considered to be unsatisfactory for the separation of the peptides obtained from capreomycin.

Still another approach was tried to obtain pure peptides. Capreomycin was hydrolyzed at 70° for 65 hr in concentrated hydrochloric acid, and the peptides obtained were converted into their DNP-ethyl ester derivatives and extracted into ethyl acetate. It was believed that peptides containing capreomycidine would not be extracted into ethyl acetate because of the presence of the quanidino group in such peptides. Several of these ethyl acetate-soluble DNP-compounds were isolated using chromatography over silicic acid followed by preparative TLC. Through interpretation of the mass spectra of these compounds it was possible to identify some of the peptides released from capreomycin upon acid hydrolysis.

Interpretation of the Mass Spectra of the DNP-Ethyl Ester Derivatives Obtained from Partial Hydrolysis of Capreomycin

It was expected that the mass spectra of DNP-peptide esters would exhibit some of the typical fragmentations of peptides. A typical peptide (formula X) undergoes fragmentation in the mass spectrometer by cleavage of bonds 1 or 2, or occasionally of bond 3. In such cleavages the positive charge can be retained by either one of these fragments formed (46).

It was also expected that some of the characteristic ions observed for DNP-amino acid esters would be present in the mass spectra of DNP-



X

peptide esters containing the same DNP-amino acids. For the interpretation of the mass spectra of DNP-peptide esters obtained from capreomycin it was necessary to have mass spectra of the DNP-ester derivatives of all the amino acids known to occur in capreomycin.

The mass spectra obtained for bis-DNP-β-lysine ethyl ester and ε-mono-DNP-β-lysine ethyl ester have been discussed previously. The mass spectrum of DNP-alanine methyl ester was also known from previous work (38). Since it was expected that DNP-capreomycidine, like other quanidino containing amino acids, would not give a useful mass spectrum (39) no attempt was made to obtain a mass spectrum of this compound. As explained before, the mixture of DNP-peptide esters was worked up in such a way as to exclude any capreomycidine containing compounds. The lack of a mass spectrum of DNP-capreomycidine was, therefore, not thought to present a hindrance in the interpretation of the spectra of the DNP-peptide esters.

The only remaining DNP-amino acid ester of which a mass spectrum was needed was the bis-DNP-diaminopropionic acid ester. This compound was prepared by esterification of bis-DNP-diaminopropionic acid and its mass spectrum was recorded (compound 13, Table 7). By using the spectra of these known DNP-amino acid derivatives as reference and by considering the general pattern of fragmentation of peptides. the mass spectra of

the compounds in Table 7 can be interpreted.

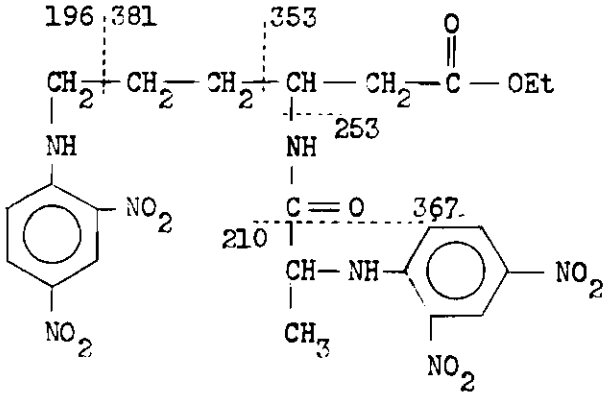
In the mass spectrometer compound 1 gave amongst others, ions with m/e values of 196, 282, and 419. These ions were characteristic of the fragments arising from amine-type cleavages involving both nitrogens of bis-DNP-β-lysine ethyl ester. Further confirmation that this compound was bis-DNP-β-lysine ethyl ester was provided by an abundant ion with m/e 322 arising through the loss of 184 mass units (2,4-dinitroaniline plus one hydrogen) from the molecular ion.

The mass spectrum of compound 2 showed an ion at m/e 183 indicating that 2,4-dinitroaniline was eliminated from this compound. This suggested that the material was a DNP-derivative of an amino acid or peptide. The absence of ions associated with DNP-derivatives of β-lysine, diaminopropionic acid, and alanine made it impossible to identify this compound from its mass spectrum only.

Ions at m/e 205 and m/e 208 in the mass spectrum of compound 3 suggested that this compound was bis-DNP-diaminopropionic acid ethyl ester or a peptide containing bis-DNP-diaminopropionic acid. In addition to these ions a characteristic ion observed for bis-DNP-diaminopropionic acid methyl ester was the one resulting from the elimination of 2,4-dinitroaniline from the molecular ion. The ion at m/e 281 observed for compound 3 could also be explained in terms of an elimination of 2,4-dinitroaniline from the molecular ion. This ion confirmed that compound 3 was bis-DNP-diaminopropionic acid ethyl ester.

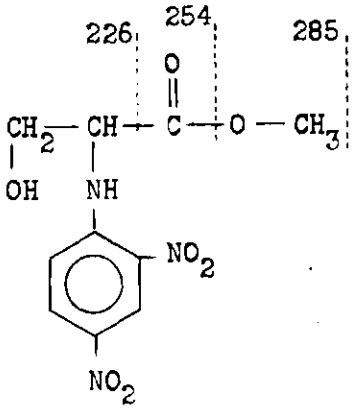
The mass spectrum of compound 4 showed an ion at m/e 210 which suggested that a DNP-alanine derivative was present. An ion at m/e 196 indicated that either the ε-amino group of a β-lysine or the β-amino

group of a diaminopropionic acid in this compound was substituted with a 2,4-dinitrophenyl group. The ion at m/e 322 indicated that compound 4 contained a DNP-derivative of β-lysine ethyl ester. This ion originated in bis-DNP-β-lysine ethyl ester from the elimination of 2,4-dinitroaniline (a mono substituted ammonia derivative) plus one hydrogen from the molecular ion. Elimination of DNP-alanine-amide (also a mono substituted ammonia derivative) and one hydrogen from ε-DNP-β-(DNP-alanyl)-β-lysine ethyl ester (formula XI) would also give rise to a fragment with m/e 322. Further confirmation that compound 4 was indeed ε-DNP-β-(DNP-alanyl)-β-lysine ethyl ester was provided by ions at m/e 353, m/e 253, m/e 367 and m/e 381. Formula XI shows how these fragments could be formed.



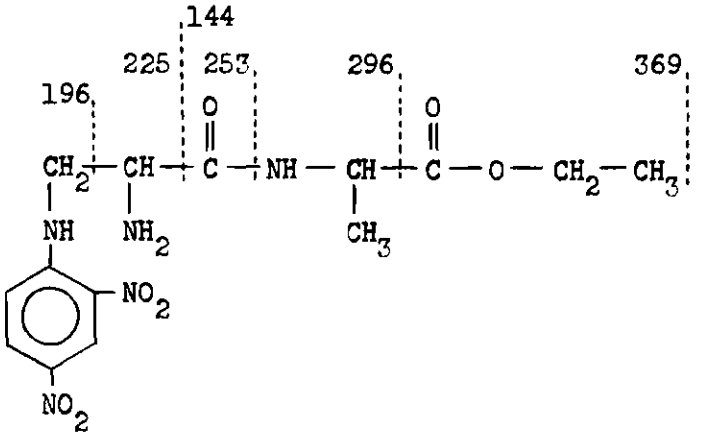
XI

Compound 5 was identified as the ethyl ester of DNP-serine (formula XII) by means of its molecular ion and by the cleavages involving the α-amino group.



XII

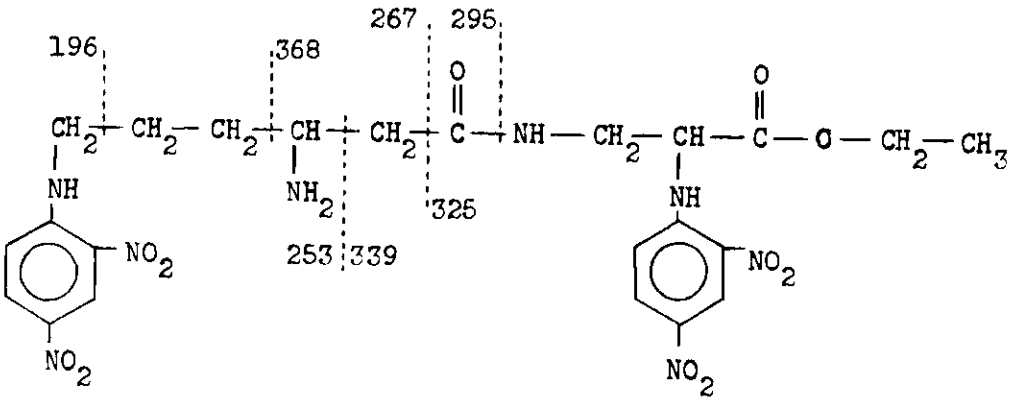
The ion with the highest m/e value in the mass spectrum of compound 6 was found at m/e 369. This corresponded to the molecular ion of a mono-DNP-derivative of diaminopropionylalanine ethyl ester. An ion at m/e 196 indicated that the β-amino group of the diaminopropionyl unit was substituted with a 2,4-dinitrophenyl group. Other ions which were important for the elucidation of the structure of compound 6 and the way in which they were thought to originate are shown in formula XIII.



XIII

It was not possible from the mass spectrum alone to arrive at a structure for compound 7.

The mass spectrum of compound 8 showed ions with m/e values of 196, 253, 267, and 295, which indicated the presence of a ε-DNP-β-lysine unit. Ions with m/e values of 368, 339, and 325 suggested, as shown in formula XIV, that the β-lysine unit was linked to a mono-DNP-diaminopropionic acid ethyl ester. An indication that a DNP-diaminopropionic acid ethyl ester group was present in compound 8 was also supplied by the ions at m/e 205 and m/e 208.



XIV

In the case of DNP-diaminopropionic acid methyl ester, it was observed that elimination of 2,4-dinitroaniline from the molecular ion took place. Apparently both fragments formed in such an elimination could retain the positive charge, since ions at m/e 183 and m/e 267 were observed. If compound 8 was indeed ε-DNP-β-lysyl-DNP-diaminopropionic acid ethyl ester, elimination of ε-DNP-β-lysine amide would yield ions at m/e 311 and m/e 281. Both these ions were observed, presenting further proof that compound 8 had the structure depicted in

formula XIV. From this mass spectrum alone it was not possible to determine which nitrogen of the diaminopropionic acid unit contained the DNP- substituent.

The mass spectra of compounds 9 through 12 could not be interpreted.

A mass spectrum of bis-DNP-diaminopropionic acid methyl ester was recorded as compound 13. As already mentioned, elimination of 2,4-dinitroaniline from the molecular ion was responsible for ions at m/e 267 and m/e 183. Loss of a carboethoxy group from the ion at m/e 267 could explain an ion observed at m/e 208 while loss of nitric oxide from the m/e 267 ion could have been responsible for an abundant ion observed at m/e 237. Another ion, characteristic of this compound in the high mass region, was observed at m/e 205. It was not possible to explain the origin of this ion.

One of the two β-lysine units in capreomycin was found to be N-terminal. It was therefore conceivable that partial acid hydrolysis of DNP-capreomycin would yield bis-DNP-β-lysine-containing peptides. Since such bis-DNP-peptides would come only from the N-terminal β-lysine it should be possible to distinguish between peptides originating from the N-terminal β-lysine and peptides containing the non-terminal β-lysine.

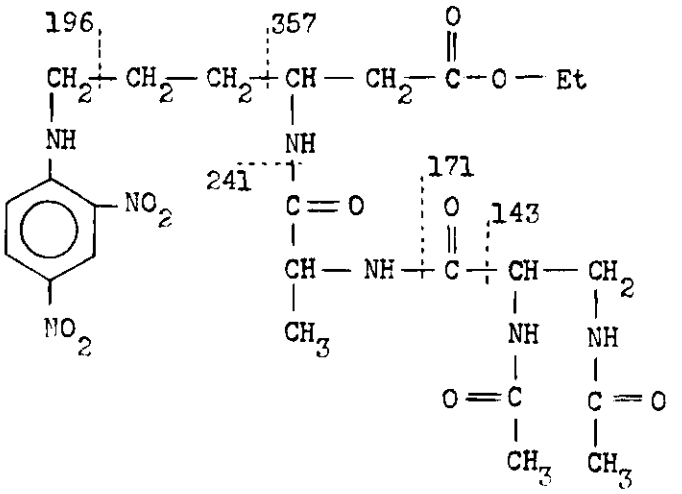
DNP-capreomycin was subjected to partial acid hydrolysis, and the hydrolysate was esterified with ethanol/hydrogen chloride. The reaction mixture was then reacted with acetyl chloride and was chromatographed over silicic acid with chloroform. A mass spectrum was taken of the first yellow band that was eluted (compound 14).

An ion at m/e 196 in the mass spectrum of compound 14 indicated that this compound had an ε-DNP-β-lysine unit. An ion at m/e 322, previously noted to originate from the elimination of a substituted ammonia plus a hydrogen from the molecular ion of DNP-β-lysine ethyl ester derivatives, was also observed for compound 14. These two ions indicated that compound 14 was a β-substituted -DNP-β-lysine ethyl ester. Alanine was found to be linked to the β-amino group of β-lysine in compound 4. It was also found that diaminopropionic acid was linked to alanine in compound 6. Considering this, and by recognizing that compound 14 represents an acetylated compound, it was thought that compound 14 might be diacetyldiaminopropionylalanyl-(ε-DNP)-β-lysine ethyl ester (formula XV). Ions which support this structure were observed at m/e 357 and at m/e 241. Ions which were not reported in Table 6 because they occurred in the mass region below m/e 180 but which also seem to support the structure proposed for compound 14 (formula XV), were observed at m/e 143 and m/e 171.

The ammonia observed to be eliminated from lysine esters reportedly could originate from any one of the two amino groups present in lysine (43). It was, therefore, also conceivable that in case of a di-substituted β-lysine derivative the elimination of a substituted ammonia could similarly originate from any one of the two substituted amino groups.

As already mentioned, the elimination of diacetyldiaminopropionylalanyl amide (the β-substituent) and a hydrogen from the molecular ion of compound 14 was thought to give rise to the ion at m/e 322. Elimination of 2,4-dinitroaniline (the ε-substituent) and a hydrogen from the

molecular ion of diacetyldiaminopropionyl-alanyl-(ε-DNP)-β-lysine ethyl ester would give rise to an ion with m/e 397. The fact that this ion was indeed very abundant in the mass spectrum of compound 14 supplied additional support that formula XV was the correct structure of this compound.



XV

The fact that the ε-amino group in this peptide was found to be substituted with a DNP-group shows that, in addition to the free amino groups of one β-lysine unit, the ε-amino group of the second β-lysine unit in capreomycin IB was free also.

The Hydrogenation of Capreomycin

Capreomycin (0.0045 moles)* was hydrogenated at atmospheric pressure in the presence of platinum. This reaction consumed 0.018

*The molecular weight of capreomycin was taken as 1005.

moles of hydrogen over a period of three weeks before it was complete.

An investigation showed that the chromophore of capreomycin was absent in the hydrogenated material and also that in the course of the hydrogenation reaction, urea was released. It was also found that the proton at τ 1.9 in the nmr spectrum of capreomycin was absent in hydrogenated capreomycin.

In an effort to find out more about the changes hydrogenation brought about in capreomycin, the perhydrocapreomycin was hydrolyzed in acid and the amino acids formed were separated quantitatively on a tall Dowex-50 X-8 column. The ratios of amino acids found in hydrogenated capreomycin was alanine: diaminopropionic acid: β-lysine: capreomycidine:: 2.22: 1.98: 1.16: 1.00. From these ratios it was apparent that hydrogenated capreomycin contained one more mole of alanine than was found in capreomycin. Furthermore, the results indicated that in hydrogenated capreomycin diaminopropionic acid, β-lysine, and capreomycidine were present in the ratios 2:1:1. These ratios were also observed in capreomycin IB by Herr and Redstone (8). In this investigation the ratio of these amino acids was found to be 3:2:1. As was previously explained, the resistance of the dipeptide β-lysyl-diaminopropionic acid toward acid hydrolysis might account for the difference observed between the different amino acid analyses. Apparently the conditions used for the hydrolysis of hydrogenated capreomycin (6 N hydrochloric acid on a steam bath for 36 hr) was not sufficient to hydrolyze this peptide, hence the low values of β-lysine and diaminopropionic acid.

Any structure proposed for capreomycin will have to take in

account the sterochemistry of the amino acids in the molecule. Since it was thought that the hydrogenation process would not affect the optical active centers of the amino acids, the sterochemistry of the amino acids isolated from hydrogenated capreomycin was determined by measurement of their optical rotations.

Alanine from hydrogenated capreomycin had a $[M]_D$ value of +16.6 ($[\alpha]_D = +18.7^o$) in 5 N hydrochloric acid whereas the $[M]_D$ value reported in the literature (44) for L - alanine was $+13.0^o$. It was, therefore, concluded that both the alanine originally present in capreomycin and the alanine formed during the hydrogenation process were L-alanine.

Diaminopropionic acid isolated from hydrogenated capreomycin had $[M]_D = +22.5^o$ ($[\alpha]_D = +21.6^o$) while the $[M]_D$ value reported in the literature for L-diaminopropionic acid was $+35.4^o$ (44). It therefore appeared that the diaminopropionic acid in capreomycin was the L-configuration.

β-lysine from hydrogenated capreomycin had a $[\alpha]_D$ value of $+19.2^o$ in 5 N hydrochloric acid compared to a $[\alpha]_D$ value of $+24^o$ reported for L-β-lysine in 1 N hydrochloric acid (47). It appeared therefore that the β-lysine in capreomycin was also L-β-lysine. The absolute stereochemistry of capreomycidine was previously reported (18).

It is interesting to note that capreomycidine consumed four equivalents of hydrogen during the hydrogenation reaction. It is possible that the release of urea involved a hydrogenolysis reaction which would account for one mole of hydrogen. Reduction of a dehydroalanine derivative to alanine would have consumed another mole of hydrogen. It is, however, also interesting to note that the additional mole of alanine

produced by the hydrogenation reaction appeared to be L-alanine. Reduction of a dehydroalanine derivative of the kind proposed to be responsible for the chromophore in viomycin and capreomycin (25) would not be expected to proceed in such a stereospecific way.

The fact that urea is released during hydrogenation and also that an additional alanine was formed does not necessarily mean that these groups were involved in the chromophore. It has to be remembered that two more moles of hydrogen were consumed which could have been responsible for the destruction of the chromophore.

The Reaction of Capreomycin with Cupric Ions

An X-ray crystallographic investigation would be an ideal way to determine the structure of capreomycin. It is, however, necessary to have a crystal of a derivative of capreomycin that contains a heavy atom for such an investigation. In an effort to obtain such a crystalline derivative, capreomycin was treated with copper (II) hydroxide to form a copper complex. This complex was chromatographed over Sephadex G-15 to obtain material which appeared to be pure according to TLC and electrophoresis analyses. All attempts to crystallize this copper complex were unsuccessful.

The amount of copper present in a sample of the complex was determined by means of titration with EDTA and a molecular weight of 1125 for the copper complex was estimated from the titration data. This molecular weight compared favorably (within the limits of experimental error) with the apparent molecular weight of 1005 reported for capreomycin IB.

CHAPTER IV

CONCLUSIONS

Upon acid hydrolysis capreomycin IB releases ammonia, alanine, diaminopropionic acid, β-lysine, and capreomycidine in the ratio 2:1:3:2:1. Urea is also released from this antibiotic by acid hydrolysis.

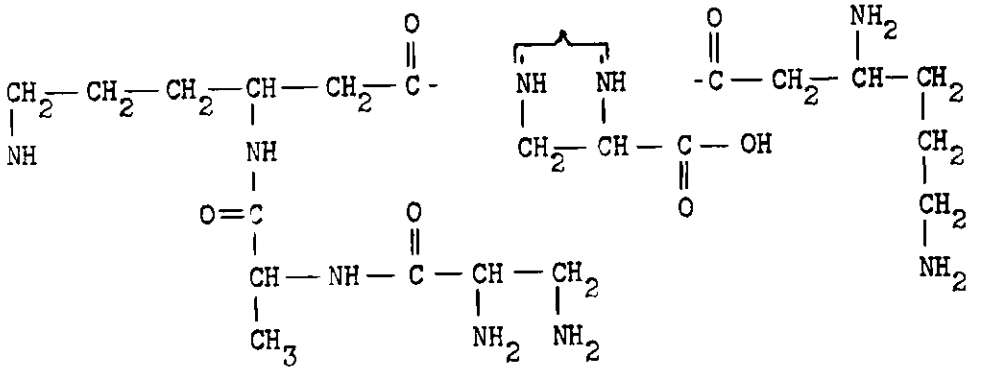
Hydrogenation of one equivalent of capreomycin required four equivalents of hydrogen. During this reaction the uv chromophore of capreomycin disappears and urea is released from the molecule. Acid hydrolysis of the hydrogenated material released two equivalents of alanine, indicating the presence of a dehydroalanine derivative or a fragment that can be converted to alanine through hydrogenolysis. End-group analyses on perhydrocapreomycin revealed that this alanine derivative is neither N-terminal nor C-terminal.

End-group analyses on capreomycin show that one of the two β-lysine units in this compound is N-terminal. Isolation of the peptide diaminopropionyl-alanyl-(ε DNP)-β-lysine from a partial acid hydrolysate of DNP-capreomycin prove that the ε-amino group of the second β-lysine unit is also free in capreomycin. The C-terminal fragment does not correspond to any of the amino acids known to occur in capreomycin.

End-group analysis on desureacapreomycin shows that removal of urea from capreomycin does not create a new C-terminal amino acid, and that urea is, therefore, not bonded as a ureide in this compound.

The peptides alanyl-β-lysine, β-lysyl-diaminopropionic acid, diaminopropionyl-alanine, and diaminopropionyl-alanyl-β-lysine occur in capreomycin. Alanine is bonded to the β-amino group of β-lysine. It is not known which one of the two amino groups of diaminopropionic acid is involved in the peptide linkage with β-lysine in the peptide β-lysyl-diaminopropionic acid.

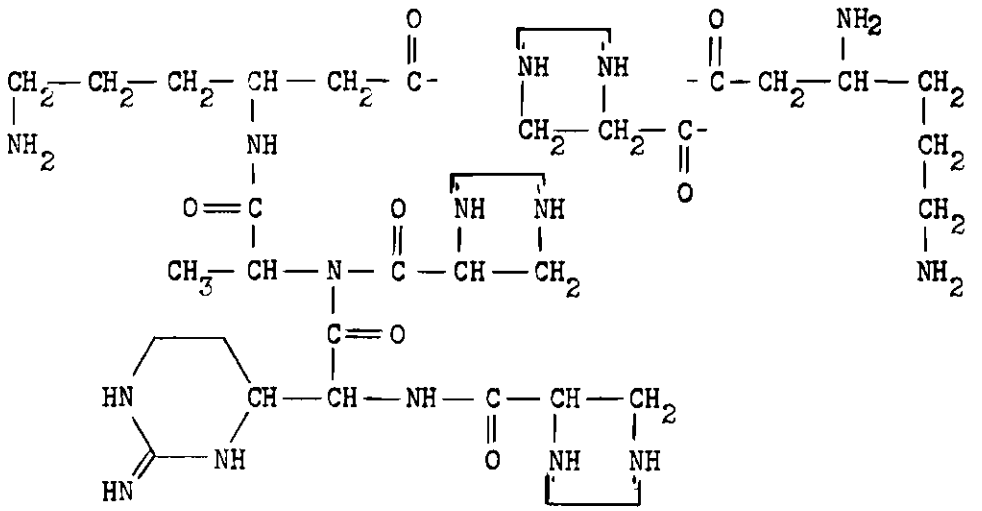
As indicated in formula XVI, it is not possible from the available data to determine which one of the β-lysine units is bonded to diaminopropionic acid.



XVI

It is interesting to note that the presence of the peptide capreomycidyl-alanine in capreomycin IB was reported by Herr and Redstone (8), whereas in this research the amino group of alanine was found to be bonded to diaminopropionic acid. It might be that the carboxyl groups of both capreomycidine and diaminopropionic acid are bonded to the amino group of alanine. The peptide diaminopropionylcapreomycidine was also found in capreomycin IB (8,15). Combining these peptides with the peptides found in this research,

it is possible to propose the partial structure XVII for capreomycin.



XVII

Formula XVII does not indicate which one of the β-lysine units is bonded to diaminopropionic acid. It also does not show which one of the three diaminopropionic acid units is involved in this linkage with β-lysine. Further investigation of the DNP-peptides resulting from the partial hydrolysis of DNP-capreomycin should resolve this problem.

It is not known where in the peptide sequence the dehydroalanine derivative occurs. Partial acid hydrolysis of hydrogenated capreomycin should yield alanine-containing peptides which should give more information on this question.

The way in which urea is bonded to the rest of the molecule and the nature of the chromophore in capreomycin are still unknown.

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The author was married to Maria Elizabeth Grobler on July 31, 1965.